REMARKS

Applicants note certain formal errors in Applicants' previous response filed April 2, 2007, and in the Office Action mailed June 12, 2007. In the response filed April 2, 2007, Applicants made certain amendments to the claims that were not identified by underline and strike-through as required. Applicants apologize for this error. The remarks in the Office Action mailed June 12, 2007 appear to recognize the amendments to the claims in some instances, but not in others. Further, that Office Action states that it is "in response to the communication filed 6-30-06." See Office Action at page 2. That is the date of the previously filed response. The Office Action also refers to "Applicant's arguments filed 10-2-06." Office Action at pages 2 and 4. That is the mailing date of the previous Office Action. Thus, it is not entirely clear whether the Examiner considered and/or entered the amendments made in the response of April 2, 2007. To avoid further confusion, Applicants have canceled the previously pending claims without prejudice or disclaimer and have added new claims. Upon entry of this amendment, claims 108-119 will be pending. Support for new claims 108-119 can be found throughout the specification as filed, for example at paragraph 000197, and at original claims 90-94. Thus, the amendment adds no new matter.

Applicants respectfully submit that the rejections to claims 90, 93, and 94 should not be applied to new claims 108-119.

35 U.S.C. § 112, first paragraph (written description)

The Examiner rejected claims 90, 93, and 94 under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter that was not described in the specification in such a way as to reasonably convey to a skilled artisan that the inventors had possession of the claimed subject matter at the time of filing the application. See Office Action at page 2. Applicants respectfully traverse and request that the rejection not be applied to new claims 108-119.

The Examiner asserted that the "specification, claims and the art do not adequately describe features or attributes concisely shared by the members of the genus comprising these compounds that specifically hybridize and successfully elicit cleavage in any target in a cell in vitro or in vivo." Office Action at page 3. Applicants respectfully disagree.

Claim 108 recites methods comprising contacting a cell with an oligomeric compound comprising a single-stranded oligonucleotide consisting of 12 to 30 linked nucleosides wherein the

oligonucleotide has a nucleobase sequence complementary to the nucleobase sequence of the target RNA, each nucleoside of the oligonucleotide comprises a 2'-fluoro modification; and at least one internucleoside linkage of the oligonucleotide is a phosphorothioate linkage. The claim thus does recite a genus of compounds that share certain common features. The Examiner seems to suggest that the claim ought to be limited to methods wherein the target RNA is PTEN messenger RNA. While Applicants agree that the specification provides written description support for such methods, Applicants respectfully submit that there is no reason why the claims should be limited to that target RNA. Indeed, PTEN messenger RNA is included merely as an example of a target that may be used in the recited methods using oligonucleotides comprising certain motifs. It is the motifs and not the target that provide the basis for the claimed genus. One of ordinary skill in the art will certainly recognize that an oligomeric compound complementary to any target RNA can be made according to the recited motifs.

As applicants pointed out in the previous response, the written description requirement does not necessitate an exhaustive listing of every embodiment that falls within the scope of the claims when a generic description is provided. Claim 108 recites methods of eliciting cleavage of a target RNA using an oligomeric compound comprising a single-stranded oligonucleotide having a defined motif. Applicants respectfully submit that claim 108 fully satisfies the written description requirement of § 112. Each of claims 109-117 ultimately depends from claim 108 and, thus, each of those claims, likewise satisfies the written description requirement. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph not be applied to new claims 108-124.

35 U.S.C. § 112, first paragraph (enablement)

The Examiner rejected claims 90, 93, and 94 under 35 U.S.C. § 112, first paragraph as allegedly not being enabled by the specification. Applicants respectfully disagree and request that this rejection not be applied to new claims 108-119.

As discussed in the previous response, an application enables the claims "if one skilled in the art, after reading the disclosure, could practice the invention claimed . . . without undue experimentation." *Chiron Corp. v. Genentech, Inc.*, 363 F.3d 1247, 1253 (Fed. Cir. 2004). "But the question of undue experimentation is a matter of degree. The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation 'must

not be unduly extensive.'" *PPG Indus., Inc. v. Guardian Indus., Corp.*, 75 F.3d 1558, 1564 (Fed. Cir. 1996) (quoting *Atlas Powder Co. v. E.I. DuPont de Nemours & Co.*, 750 F.2d 1569, 1576 (Fed. Cir. 1984)).

Applicants respectfully submit that at the time of filing, it was within the skill of the art to identify a target, the cleavage of which is desired. Next, it was within the skill of the art to design and synthesize oligonucleotides complementary to such a target. With guidance from the specification, it was within the skill in the art to incorporate the chemical modifications recited in the claims. And, finally it was within the skill in the art to screen such oligonucleotides for the desired cleavage. Applicants submit that such process is analogous to the making and screening of monoclonal antibodies, which the Federal Circuit found not to be undue experimentation in 1980 in *In re Wands*:

> This process entails immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics.

Id at 1407. The Examiner asserts that the "quantity of experimentation required to practice the invention as claimed would require the de novo determination of a representative number of antisense oligonucleotides 12-30 nucleobases in length targeted to the RNA." Office Action at page 6. As the above example indicates, such experimentation need not render an invention non-enabled.

In rejecting the previously pending claims, the Examiner asserted that "the art is quite unpredictable regarding the ability to elicit cleavage of any target messenger RNA in a cell in vivo using the broad genus of compounds claimed." Office Action at page 6. Applicants respectfully disagree. An application must be enabled at the time of filing. By September 18, 2002, (the filing date of the provisional application to which the instant application claims priority) the art was replete with evidence that one of ordinary skill in the art could design, synthesize, and screen oligonucleotides complementary to a desired target RNA to identify a sequence that would elicit cleavage. For example, a review article published in June of 2002 remarks that:

> Typically, we evaluate between 20 and 80 oligonucleotides in the primary screen for oligonucleotides that work by an RNase H-dependent mechanism of action. This number is selected not because it requires examination of 20-80 oligonucleotides to identify an antisense lead but because examining this number of

> oligonucleotides generally provides several high-quality leads for use in pharmacology model systems, and further optimization often is not required.

Bennett CF, *Antisense & Nucleic Acid Drug Development*, 12(3):215-224 (June, 2002) (copy enclosed as Appendix A). Moreover, certain considerations in designing oligonucleotide sequences may be found in the art of the time, for example in *Antisense Drug Technology, Principles, Strategies, and Applications*, Edited by S.T. Crooke, Marcel Dekker, Inc., New York, NY (2001), at Chapter 5: Methods of Selecting Target Sites in RNA for Antisense Targeting by S.M. Freier (copy enclosed as Appendix A). Thus, at the time of filing, one of ordinary skill in the art could follow the teachings of Freier to design oligonucleotides complementary to a target RNA. It was well within the ordinary skill to synthesize and screen 20-80 such oligonucleotides, which according to Bennett would likely result in several high-quality leads. One of ordinary skill in the art could thus have prepared and confirmed the activity of antisense sequences without undue experimentation.

The Examiner also remarked that in addition to identifying a suitable target sequence, to practice the claimed invention, one would have to determine "modes of delivery and formulations to target appropriate cells and/or tissues in an organism, whereby the compound or compounds are effectively delivered in adequate quantities to the target cells and cleavage of any target RNA is elicited." Office Action at pages 6-7. However, the specification demonstrates that certain oligonucleotides do posses *in vivo* activity. Although the oligonucleotides tested in the *in vivo* studies reported in Example 13 (pages 51-53) do not comprise the presently claimed motifs, compounds that do fall within the scope of the claims were shown to have potent activity in cells. See e.g., ISIS 319022 on page 51. Moreover, the link between *in vitro* results and *in vivo* activity for oligonucleotides was established at the time of filing. See e.g., Butler et al, *Diabetes,* 2002:51, 1028-1034 (reporting potent antisense inhibitor of PTEN in 3T3L1 adipocytes (see page 1029, second column, first paragraph) substantially reduces PTEN mRNA and protein when administered via intraperitoneal injection to mice (see Figure 2)); and Zinker, et al., *PNAS*, 2002:99 (17) 11357-22362 (showing *in vitro* antisense inhibitor of PTP1B mRNA expression (see page 11357, penultimate paragraph) inhibits PTP1B mRNA and protein when administered via intraperitoneal injection to mice (see page 11359, first paragraph and Figure 1)) (copies enclosed as Appendix A).

Thus, it was within ordinary skill in the art at the time of filing to (1) select a target RNA, (2) synthesize one or more oligomeric compounds having a sequence complementary to the target RNA and comprising the claimed motif, (3) screen such oligomeric compounds in cells, and (4) contact a cell with such compounds to elicit cleavage of the target RNA, including instances where the cell is in an animal, without undue experimentation.

Applicants respectfully submit that the specification provides sufficient guidance to enable one of ordinary skill in the art to fully practice the claimed invention requiring only routine experimentation. Thus, Applicants respectfully request that the Examiner not apply the rejection of claims 90, 93, and 94 under 35 U.S.C. § 112, first paragraph to new claims 108-119.

35 U.S.C. § 103(a)

The Examiner rejected claims 90, 93, and 94 as allegedly obvious under 35 U.S.C. § 103(a). In particular, the Examiner asserted that claims 90, 93, and 94 are "unpatentable over Fire and Zamore et al. and Elbasher et al., in view of McKay." Office Action at page 9. Applicants respectfully traverse this rejection because the presently claimed methods would not have been obvious to those of ordinary skill in the art at the time of applicants' invention.

According to the Supreme Court, a finding of obviousness requires identification of "a **reason** that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR Int'l Co. v. Teleflex*, 127 S.Ct. 1727 (2007) (emphasis added). The KSR Court further noted that the analysis underlying the obviousness determination "should be made explicit." *Id.* at 1741.

In applying the legal principles of KSR to a case involving chemical compounds, the Federal Circuit held that "it remains necessary to identify some **reason** that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound." *Takeda Chemical Industries, LTD v. Alphapharm Pty, Ltd.*, 83 USPQ 2d 1169, 1174 (Fed. Cir. 2007) (emphasis added). As previously held by the Federal Circuit and reiterated by the KSR Court, "rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated **reasoning** with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn*, 441 F.3d 977, 988 (CA Fed. 2006) (emphasis added). The U.S. Patent and Trademark Office recently promulgated guidelines for Examiners in making

obviousness determinations in view of the U.S. Supreme Court's decision in *KSR Int'l Co. v. Teleflex Inc.* Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR International Co. v. Teleflex Inc., 72 Fed. Reg. 57,526 (2007) ("Guidelines"). One important feature of the Guidelines is an *explicit requirement* that an Examiner provide articulated reasons for the factual determinations underlying an asserted *prima facie* case of obviousness. This focus is consistent with the rule set down in the KSR decision that a fact-finder must provide "reasons" why an invention would have been obvious to one of ordinary skill in the art. ." *KSR* at 1741. In explicating this aspect of the Supreme Court's decision, the Guidelines set forth explicit factual findings that an Examiner must articulate to support an obviousness rejection. For an obviousness rejection based on a rationale of combining references, the Examiner *is required to articulate* the following: (1) a finding that the prior art included each element claimed; (2) that one of ordinary skill in the art could have combined the elements by known methods, and that in combination each element merely would function as it did separately; (3) one of ordinary skill in the art would have recognized that the results of the combination were predictable; and (4) whatever additional findings based on the Graham factual inquiries may be necessary, in view of the facts of the case under consideration, to explain a conclusion of obviousness. Fed. Reg. at page 57,529.

Applicants respectfully submit that the cited references, when considered individually or in combination, fail to teach or suggest every limitation of the present claims. Moreover, there is no reason why those of ordinary skill in the art would have modified the teachings of the cited references to arrive at the presently claimed subject matter. The Fire, Zamore, and Elbashir references each fail to teach or suggest oligonucleotides that comprise a 2'-fluoro modification at each nucleoside. Although the McKay reference describes oligonucleotides that do comprise 2'-fluoro modified nucleosides, the reference fails to teach or suggest oligonucleotides in which *each* nucleoside comprises a 2'-fluoro group, as presently claimed. The cited references thus fail to describe, teach, or suggest every limitation of the claims.

Moreover, those skilled in the art would not have modified the teachings of the cited references to arrive at the presently claimed subject matter in light of the teachings of the cited art. In the previous response, Applicants pointed out that Fire, Zamore, and Elbashir all teach using double-stranded oligonucleotides. Indeed, the Fire reference reports that double-stranded RNA is 100-fold more effective than single-stranded RNA. The present office action asserts that such 100-fold decrease in

efficacy does not teach away from the use of single-stranded RNA. Applicants respectfully disagree. The Office has failed to articulate a credible reason why one of ordinary skill in the art would have modified and used a type of compound that has been reported to be 100-fold less effective than other types of compounds.

Furthermore, the Elbashir reference reports that 2'-modification of every nucleoside of an oligonucleotide *abolished* the oligonucleotide's activity. Elbashir at page 6882, first full sentence. Accordingly, based upon the teachings of the Elbashir reference, those skilled in the art would not have reasonably expected a fully 2'-modified oligonucleotide to have been active. The Office has failed to articulate a credible reason why one of ordinary skill in the art would have modified an oligomeric compound using a motif that had been reported to abolish activity. Together, the Fire and Elbashir references discourage one of skill in the art from producing and using either fully modified or single-stranded oligonucleotides, both of which are recited in the present claims.

Since the cited references fail to describe, teach, or suggest every limitation of the present claims, and since there is no reason why those of skill in the art would have modified the teachings of the references to arrive at the presently claimed subject matter, such subject matter would not have been obvious to those of ordinary skill in the art at the time of the invention. Applicants accordingly submit that the present rejection does not apply to the subject matter of new claims 108 to 119, and respectfully request withdrawal thereof.

CONCLUSION

Applicants believe that all outstanding issues in this case have been resolved and that the present claims are in condition for allowance. Nevertheless, if any undeveloped issues remain or if any issues require clarification, the Examiner is invited to contact the undersigned at the telephone number provided below in order to expedite the resolution of such issues.

Respectfully submitted,

Date: November 13, 2007

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APPENDIX A

ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT 12:215–224 (2002)

Review

Efficiency of Antisense Oligonucleotide Drug Discovery

C. FRANK BENNETT

ABSTRACT

The costs for discovering and developing new drugs continue to escalate, with current estimates that the average cost is more than $800 million for each new drug brought to the market. Pharmaceutical companies are under enormous pressure to increase their efficiency for bringing new drugs to the market by third-party payers, shareholders, and their patients, and at the same time regulators are placing increased demands on the industry. To be successful in the future, pharmaceutical companies must change how they discover and develop new drugs. So far, new technologies have done little to increase overall efficiency of the industry and have added additional costs. Platform technologies such as monoclonal antibodies and antisense oligonucleotides have the potential of reducing costs for discovery of new drugs, in that many of the steps required for traditional small molecules can be skipped or streamlined. Additionally the success of identifying a drug candidate is much higher with platform technologies compared to small molecule drugs. This review will highlight some of the efficiencies of antisense oligonucleotide drug discovery compared to traditional drugs and will point out some of the current limitations of the technology.

INTRODUCTION

DISCOVERY AND DEVELOPMENT OF NEW DRUGS continue to be very expensive and inefficient processes. It is estimated that the average cost of discovering and developing a successful new drug in the United States today is approximately $800 million (*www.tufts.edu/med/csdd/images/NewsRelease113001pm.pdf*). This compares with an average cost of $231 million in 1987 and $54 million in 1976. There are several factors that contribute to these enormous costs, including the high failure rate of drugs in discovery and development, the high cost of doing research, and increased development costs.

A major contributor to the cost of drug discovery is the resources required to bring a new drug candidate to the clinic. It is estimated that an average of 250 full-time employee (FTE) years (equivalent to approximately $70 million) is required for each new chemical entity reaching the clinic (Myers and Baker, 2001). This reflects the large number of people needed for the discovery, optimization, and profiling of traditional small molecule drugs.

The high failure rate of drugs in discovery and development is another major contributor to the total cost of drug development. For the purpose of this discussion, I define drug discovery as the stage starting with an initial concept for a new target or therapeutic approach to first human dosing (Fig. 1) and development as the stage from first human dosing to regulatory approval (Fig. 2). The processes pharmaceutical and biotechnology companies use for drug discovery are undergoing marked changes, making it difficult to estimate accurately the current failure rates for the discovery stage. Combinatorial chemistry and high throughput screening processes exponentially increase the number of chemical entities and molecular targets evaluated. For the purpose of this discussion, a 10% success rate for projects that begin screening for a lead chemical to completion of investigational new drug (IND) supporting toxicology studies is a reasonable assumption. It is estimated that only 15%–20% of drugs that begin phase I clinical trials (the first phase of testing in humans) successfully make it to the market (Cockburn and Henderson, 2001). How many businesses would initiate a new project that had a 1%–2% chance of being successful? Furthermore, an investment of over $100 million may be required before it is known if the project will be successful. Given these statistics, it is remarkable that the pharmaceutical industry has survived. The historical return on investment for the few successful products has fueled the industry for the past 100 years. The high cost of drug discovery and de-

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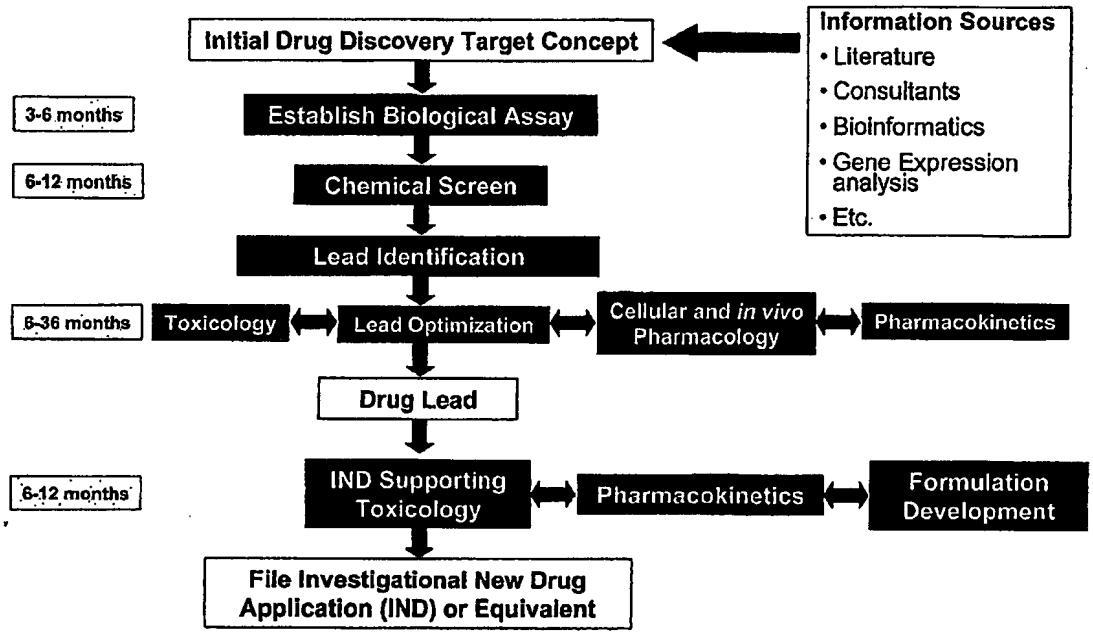

FIG. 1. Steps in traditional drug discovery.

velopment combined with increased regulatory hurdles and pressure from third party payers and governments to keep prescription medicine costs down are putting tremendous pressure on the pharmaceutical industry.

It is clear that the pharmaceutical industry must adopt changes in the discovery and development of new drugs to remain a viable industry. Historically, the pharmaceutical industry focused its resources on discovery of small molecular weight chemical entities that modulate the function of a target protein. Ideally, the molecules would be orally available and pharmacokinetics would support once-daily dosing, the "small white pill." Such drugs have been the backbone of the industry, leading to its success. The problem with such an approach is that each new chemical entity requires an enormous investment to understand sufficiently its pharmacologic, toxicologic, and pharmacokinetic properties to warrant testing in humans. Similarly, development of the manufacturing processes for each new chemical entity and establishment of the manufacturing facility require new investments. Rarely can the information learned from one small molecule drug be leveraged for the next small molecule drug. One way the industry can decrease costs and increase efficiency is to incorporate drug platform technologies into its portfolio. The advantage of platform technologies is that investments in one drug candidate can be readily leveraged for the next candidate. Examples of platform technologies include monoclonal antibodies, antisense oligonucleotides, gene therapy, and, to some extent, recombinant proteins. With the success of abciximab (ReoPro, Centocor, Inc., Malvern, PA), infliximab (Remicade, Centocor, Inc., Malvern, PA), rituximab (Rituxan, CIDEC Pharmaceuticals, San Diego, CA), and trastuzumab (Herceptin, Genintech, Inc., South San Francisco, CA), many of the major pharmaceutical companies are making investments in monoclonal antibodies as well as in recombinant protein products. Antisense oligonucleotides and gene therapy are newer platform technologies that have gone through cycles of enthusiasm, disappointment, and rational optimism. With several antisense drugs in phase III clinical trials, the pharmaceutical industry is once again beginning to make investments in the technology (Niiler, 2001).

In this review, I highlight how antisense oligonucleotides are currently contributing to increased efficiency of the drug discovery process and identify how antisense technology is a more efficient drug discovery and development technology than traditional small molecules. The review focuses primarily on preclinical drug discovery, as that is where the major efficiencies of antisense technology are realized. Development of antisense drugs requires similar resources and has similar timelines as traditional small molecule drugs, and it is assumed that the failure rate of antisense drugs in development will be similar to that of traditional small molecule drugs beyond phase I trials. Thus, the main advantages of antisense oligonucleotides are in preclinical drug discovery and early development. Antisense oligonucleotide drugs will not be the panacea that replaces all therapeutic approaches, but the technology offers some competitive advantages that make it an important drug discovery platform.

PREDISCOVERY: USE OF ANTISENSE OLIGONUCLEOTIDES FOR TARGET VALIDATION

Pharmaceutical companies are making investments in drug discovery at a much earlier stage than they have historically.

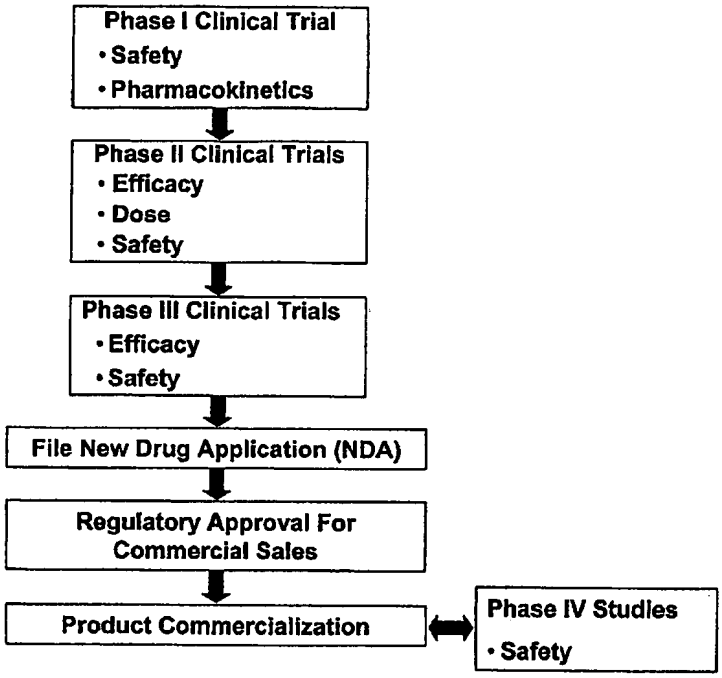

FIG. 2. Steps in drug development.

Pharmaceutical companies relied heavily on federally funded academic laboratories for novel insights into potential drug targets. With the release of large amounts of sequence information in public and private databases, the pharmaceutical and biotechnology industries have made enormous investments in genomics to protect their ability to discover drugs to novel gene products. Thus, the premise that the Human Genome Project should ultimately result in better therapies for patients is valid. However, in the short term, it has contributed to marked increases in drug discovery costs. It is also clear that far greater investments must be made to exploit the enormous information provided by the Human Genome Project for drug discovery and development. It is estimated that of the approximately 40,000 human genes, 3,000–10,000 could be potential drug targets (Drews, 1996; Reiss, 2001). Given that less than 1,000 genes have been validated as drug targets and fewer than 500 genes are targets of approved drugs, tremendous numbers of resources must yet be invested to identify which genes are appropriate targets for therapeutic intervention. Bioinformatics and gene expression data are valuable tools to narrow down the list of potential genes. However, it is ultimately critical to validate genes as potential drug targets by perturbing the function of the gene in a biologic system, ideally one that mimics human disease.

There are many technologies that can be used determine gene function in cell-based assays and validate genes as drug discovery targets in *in vivo* models, such as gene overexpression, dominant negatives, antibodies, genetic knockouts, and antisense technology. Antisense oligonucleotides are already proving to be very valuable tools for validating novel gene targets for drug discovery. The use of antisense oligonucleotides as a tool for gene functionalization and target validation has been reviewed in detail (Bennett and Cowsert, 1999a,b; Dean, 2001; Monia et al., 2001). Briefly, antisense oligonucleotides are among the quickest means to validate gene function, they offer exquisite specificity, the technology can be applied broadly toward all classes of genes and offers a seamless transfer from cell-based assays to complex *in vivo* models, and antisense technology has a high probability of working and behaves as a pharmacologic agent, that is, a drug. There have been thousands of studies published using antisense oligonucleotides to identify the function of a gene product in cell-based assays or in animal models (reviewed in Bennett and Cowsert, 1999a; Crooke and Bennett, 1996). In addition to producing a desirable effect in an experimental setting, thus validating the gene target, it is equally useful to invalidate a gene target because of lack of efficacy or unexpected toxicity resulting from inhibiting expression of the targeted gene product.

There are multiple mechanisms by which synthetic oligonucleotides can be used to regulate gene expression (Crooke, 1999). The most commonly used mechanism is through RNase H-mediated degradation of the mRNA (Agrawal et al., 1990; Chiang et al., 1991; Crouch and Dirksen, 1982; Inoue et al., 1987; Monia et al., 1993). There are additional ribonucleases that may be targeted to specific mRNAs by synthetic oligonucleotides, such as RNase L, RNase III, and the RNase associated with the RNA-induced silencing complex (RISC) (Bernstein et al., 2001; Maitra et al., 1995; Torrence et al., 1993; Wu

et al., 1998; Zamore et al., 2000). Recently, much interest has focused on small interventing RNA (siRNA) as a novel antisense strategy to functionalize genes in mammalian cell-based assays (Elbashir et al., 2001a,b; Harborth et al., 2001). Although much remains to be learned about the siRNA mechanism and its limitations, results from initial studies suggest that it is a useful tool to functionalize genes (Caplen et al., 2001; Elbashir et al., 2001a; Harborth et al., 2001; Muda et al., 2002). Many of the lessons learned about traditional antisense oligonucleotides appear to apply to siRNA.

In addition to recruiting nuclease to promote degradation of the target mRNA, oligonucleotides can be used to sterically interfere with RNA function, an example being inhibition of translation of the mRNA (Baker et al., 1997; Cazenave et al., 1989; Ghosh et al., 1992; Johansson et al., 1994; Lengyel et al., 1961; Summerton and Weller, 1997). A second example is modulation of alternative processing of the pre-mRNA that encodes the gene of interest (Dominski and Kole, 1993; Kole and Sazani, 2001; Taylor et al., 1999b; Vickers et al., 2001). Most mammalian genes produce multiple distinct mRNA species because of alternative transcription initiation, alternative splicing, and alternative polyadenylation sites. These unique RNA species may code for proteins with distinct function, as is the case for Bcl-X (Boise et al., 1993). Antisense oligonucleotides have been shown to bind to the pre-mRNA in the cell nucleus and modulate RNA maturation events (Condon and Bennett, 1996; Dominski and Kole, 1993; Sazani et al., 2001; Sierakowska et al., 1996). Thus, antisense oligonucleotides that do not promote cleavage of RNA will be valuable tools to help identify the role of different RNA variants in normal and disease processes.

Although antisense oligonucleotides are proving to be valuable tools to help determine gene function and validate genes as drug targets, like any other drug, they can produce unanticipated effects that contribute to their overall pharmacologic profile (Bennett, 1998; Lebedeva and Stein, 2001; Stein, 2001). The unanticipated effects may be due to hybridization to the RNA of an alternative gene, sequence-specific interactions with proteins (or other macromolecules), or sequence-independent interaction with macromolecules (Benimetskaya et al., 1995; Bennett et al., 1994a; Crooke and Bennett, 1996; Yakubov et al., 1993). Thus, one must use caution in interpreting results when using antisense oligonucleotides, as with any other tool. The effects of the antisense oligonucleotide can be readily validated by examining effects on expression of the targeted gene. In addition, using two or more active antisense oligonucleotides to confirm a pharmacologic effect and two or more control oligonucleotides to demonstrate sequence specificity of the effect helps build confidence that the observed biologic effect is due to inhibition of the targeted gene product.

DISCOVERY OF ANTISENSE DRUGS

Discovery of antisense drugs is a far more efficient process than discovery of traditional small molecule drugs (Figs. 1 and 3). It may take 2–6 years to identify a small molecule drug and develop the preclinical data package to allow testing in humans (Fig. 1). In contrast, it may take <1 year to develop a similar data package for an antisense drug (Fig. 3). Identification of the initial lead for a traditional small molecule drug may take 9–>18 months and requires at least 5–10 FTE equivalents during this period (Fig. 1). Even with these investments, intensive screening efforts have failed to find an adequate lead in many cases. Once the initial chemical lead has been identified, additional resources and time typically are required to optimize the chemical structure to obtain the desired potency, pharmacokinetic profile, pharmacology, and lack of toxicity. Optimization of the chemical lead to the drug product that will enter clinical trials can take from 6 months to >3 years, with 15–25 FTE dedicated to the project. In contrast, identification of an antisense oligonucleotide lead can be done in a couple of weeks, and optimization, if necessary, requires an additional couple of weeks (Fig. 3). This activity can be accomplished with 2–3 FTE over this short time.

The process for identification and optimization of antisense drugs has been described elsewhere (Baker et al., 2001; Bennett and Cowsert, 1999b). Briefly, a series of oligonucleotides (15–20 nucleotides in length) are designed to hybridize to different regions on the target mRNA or pre-mRNA. The oligonucleotides are synthesized, purified, and evaluated in a cell-based assay system that directly measures target gene expression (either RNA or protein). In that RNA forms complex secondary and tertiary structures in cells and is protein bound, not all sites on the target RNA may be accessible to the oligonucleotide. The type of antisense chemistry and desired mechanism of action will dictate which areas on the target RNA are selected for hybridization. Typically, we evaluate between 20 and 80 oligonucleotides in the primary screen for oligonucleotides that work by an RNase H-dependent mechanism of action. This number is selected not because it requires examination of 20–80 oligonucleotides to identify an antisense lead but because examining this number of oligonucleotides generally provides several high-quality leads for use in pharmacology model systems, and further optimization often is not required. If further optimization is required, one could alter oligonucleotide length, examine additional sites on the target RNA, and alter placement of any chemical modifications to further improve activity or decrease unwanted effects. Using this approach, we have had >95% success for over 2,000 genes attempted.

Antisense drugs and small molecule drugs will be evaluated in similar, if not the same, pharmacologic models. Thus, the length of time required to develop the preclinical pharmacology packages will be similar. The main advantage of the antisense approach is that there is no need to expend resources characterizing the pharmacokinetics and toxicology of the antisense drug candidate at this stage unless a new *in vivo* model is being used. In that case, it is important to establish the pharmacokinetic and pharmacodynamic relationship in the target tissue and target cells (Graham et al., 1998; Yu et al., 2001; Zhang et al., 2000). Once accomplished, the results can be applied to subsequent antisense molecules targeting different gene products in the model system.

In addition to the efficiency of antisense drug discovery, another advantage of antisense oligonucleotides is that any nuclear encoded gene is an appropriate target (there are no published reports demonstrating that antisense oligonucleotides can effectively inhibit the expression of mitochondria-encoded genes). Both traditional small molecules and monoclonal anti-

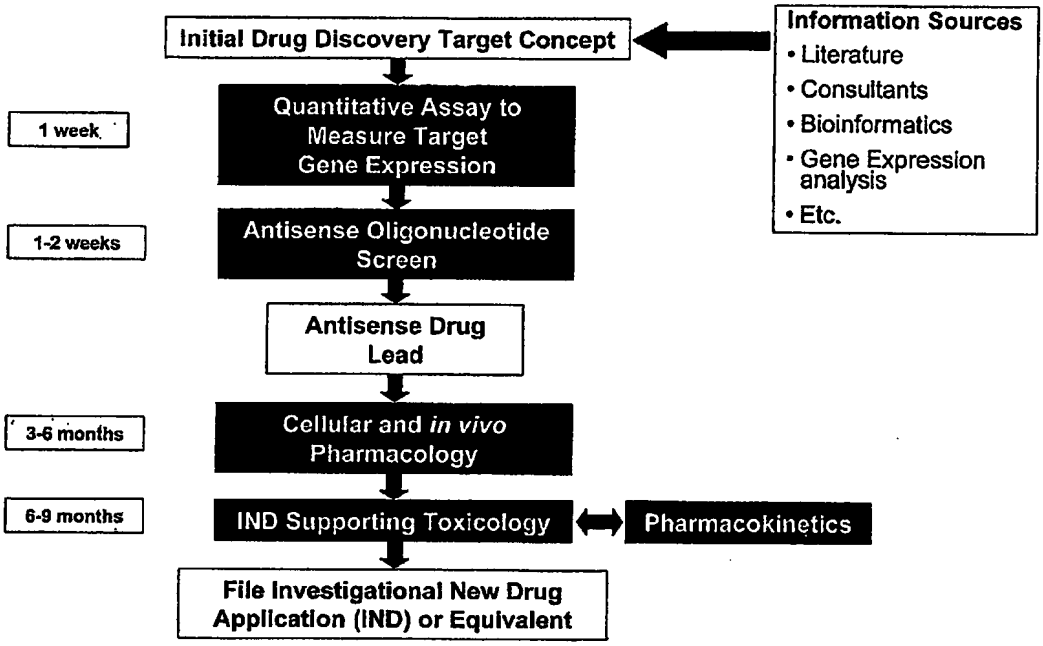

FIG. 3. Steps in antisense oligonucleotide drug discovery.

bodies currently have restrictions as to the types of genes that are amenable to therapeutic intervention. Monoclonal antibodies bind to antigens expressed on cell surfaces or in circulation. Thus, intracellular targets are not good targets for monoclonal antibody therapy. The same is true for recombinant proteins, although cell-permeating peptides may allow introduction into cells (Wadia and Dowdy, 2002).

Although small molecule-based therapeutics can approach a broader range of target genes than protein-based technology, they still have some marked limitations. In general, targets for traditional small molecule drugs are enzymes (e.g., proteases, kinases, topoisomerases, polymerases) and receptors for small molecular weight ligands (e.g., neurotransmitters, eicosanoids, steroids, retinoids). Advances in structural biology and screening technologies are increasing the number of genes approachable with small molecule drugs (Clackson and Wells, 1995; Gadek et al., 2002; Shuker et al., 1996), but success using these approaches is still limited. As a result, a large number of gene targets that are potentially important drug targets are not being exploited. We have identified selective antisense inhibitors to thousands of genes, including typical "drugable targets," such as protease, protein kinases, G-protein-coupled receptors, and cytokines, and, more importantly, proteins that are generally thought of as being poor drug targets, such as proteins involved in protein-protein interactions, transcription factors, phosphatases, phospholipid-binding proteins, and proteins of unknown function (for example, Bannerman et al., 2001; Bennett et al., 1994b; Bost et al., 1997; Butler et al., 2002; Cavarretta et al., 2002; Condon et al., 2001; Dean et al., 1994; Li et al., 1999; Miraglia et al., 1996; Taylor et al., 1999a; Urban et al., 2001; Xu et al., 1998; Zellweger et al., 2001). This is not to say that antisense oligonucleotides are the magic bullet—they do have limitations. One of the major limitations for broad exploitation of antisense oligonucleotides is pharmacokinetic, in that they do not distribute equally to all tissues and cell types within the tissues. As an example, antisense oligonucleotides do not cross the blood-brain barrier and, thus, would not be appropriate for treatment of neurologic diseases. Although there are pharmacokinetic limitations as to what diseases currently can be approached therapeutically with antisense, there do not appear to be any gene-related limitations.

PRECLINICAL TOXICOLOGY

Antisense drugs can produce several types of toxicities, such as chemical class-related toxicity (both sequence specific and sequence independent), hybridization-dependent toxicity resulting from exaggerated pharmacology, and hybridization-dependent toxicity resulting from hybridization to an ancillary gene target (Levin et al., 2001). In that the chemical class-related toxicities are similar for each antisense oligonucleotide using that particular chemistry, there is not a need to invest resources at an early stage to characterize these effects. This differs from small molecules, where each new chemical entity has its own unique set of toxicology risks and must be characterized at an early stage in the development of the molecule. Toxicity caused by exaggerated pharmacology (i.e., inhibition of the target gene) often can be detected in the pharmacology models, providing early insight into potential toxicities. This is true for both small molecule-based drugs and antisense drugs.

Far more is known about potential toxicities of first-genera-

tion phosphorothioate oligodeoxynucleotides, as the industry has vastly more experience with this class of oligonucleotides than with any other. The most prevalent class of toxicities associated with first-generation antisense oligonucleotides are chemical class-related toxicities, both sequence independent and sequence specific. First-generation phosphorothioate oligodeoxynucleotides produce anticoagulant effects (Henry et al., 1997b; Sheehan and Lan, 1998; Sheehan and Phan, 2001) and complement activation (Cornish et al., 1993; Galbraith et al., 1994; Henry et al., 1997a) through interactions with specific proteins in a sequence-independent manner. Neither of these toxicities has contributed to major clinical problems, and both can be managed by maintaining peak plasma concentrations below threshold concentrations (Dorr et al., 2001). Examples of sequence-specific nonhybridization-dependent toxicity include immune activation by oligonucleotides containing an unmethylated CpG motif (Klinman et al., 1996; Krieg, 1998; Krieg et al., 1995) and oligonucleotides that are capable of forming G-quartet structures (Bennett et al., 1994a; Burgess et al., 1995; Marchand et al., 2002; Wyatt et al., 1996; Xu et al., 2001). Once these sequence motifs are identified, they can be avoided in the design of oligonucleotide drugs.

Hybridization-dependent toxicities can occur as a result of inhibition of the target gene or serendipitous inhibition of a nontarget gene. The former may occur with any type of drug molecule, as most drugs at one dose will produce the desired therapeutic effect and at a higher dose will produce undesired toxicity. Antisense oligonucleotides also have the potential to inadvertently inhibit the expression of an RNA that was not the initial target because of sequence homology to the oligonucleotide. As already mentioned, even if the oligonucleotide has 100% homology to a second gene, it may not affect expression, as the target sequence may not be accessible to the oligonucleotide. With the completion of the sequencing of the human genome and full annotation, the risk for this occurrence can be further reduced.

Despite the wealth of information available about first-generation oligonucleotides and the increasing information available about second-generation chemistry, toxicology studies are still required before testing in humans. There is always a chance that a new oligonucleotide drug will produce a surprising toxicity not previously seen with other antisense drugs (Black et al., 1993). With proper planning, the toxicity studies can be initiated before completion of the preclinical pharmacology studies so that the toxicology studies do not cause delays in getting the new antisense drug into humans.

PRECLINICAL PHARMACOKINETICS

As discussed, the pharmacokinetic behavior of oligonucleotides is not markedly influenced by sequence. Thus, there is not a need to do extensive pharmacokinetic studies until after the drug candidate has been selected. This is a real advantage over small molecule drugs, for which rather modest modifications in the chemical structure can markedly affect pharmacokinetic behavior (Lipinski et al., 2001). Thus, it is important to characterize the pharmacokinetics of a small molecule drug during the optimization process to ensure that the final drug lead has desirable pharmacokinetic properties.

The pharmacokinetics and tissue distribution of first-generation phosphorothioate oligodeoxynucleotides have been well characterized in multiple species, including humans, and by various routes of administration (Agrawal et al., 1991; Cossum et al., 1993, 1994; Cunningham et al., 2001; Glover et al., 1997; Mehta et al., 2000; Templin et al., 1999; Yu et al., 2000; Zhang et al., 1995). The pharmacokinetics of phosphorothioate oligodeoxynucleotides have been similar across multiple species, allowing predictions of their behavior in humans. In general, they exhibit a short plasma half-life, with most of the disappearance from plasma accountable by distribution to tissues. The major distribution tissues include kidney, liver, spleen, and bone marrow (Geary et al., 2001c). The pharmacokinetics for many second-generation oligomers also have been published (Agrawal and Zhang, 1998; Agrawal et al., 1995; Crooke et al., 1996; Geary et al., 2001b; Iversen, 2001; Pardridge et al., 1995; Sandberg et al., 1999; Zhang et al., 1996). Based on these results, it is possible to predict the pharmacokinetics of a new antisense drug using the same oligonucleotide chemistry.

CONCLUSIONS

Discovery and development of new drugs are expensive and time-consuming processes. However, the tremendous benefits new medicines have provided to people more than justify the costs. To remain a viable business, it is important that the pharmaceutical and biotechnology industries lower their costs and increase their efficiency in providing differentiated drug products to consumers. It is estimated that most large pharmaceutical companies need to provide two to four new products each year to sustain the growth they have enjoyed during the last 10 years (Horrobin, 2001). There are increasing concerns that pharmaceutical companies may have difficulty doing so in the short term. Platform drug discovery technologies, such as antisense oligonucleotides, can help increase the efficiency of the pharmaceutical industries. Discovery of antisense drugs is a much more efficient process than that of traditional small molecule drugs. It is possible to discover an antisense drug and develop the data package required for filing with the FDA in less than 18 months and use approximately 20–25 FTE doing so. Compare this to a typical small molecule drug discovery program that generally requires 2–6 years to discover, optimize, and develop the data packages required for filing an IND application and uses 250 FTE (Myers and Baker, 2001). Another advantage of antisense oligonucleotides is that they can be used to inhibit the expression of molecular targets that are not easily approached with small molecule-based approaches. The probability of successfully developing a selective inhibitor to a gene product is far greater with antisense technology than with small molecules. Finally, creating the commercial manufacturing infrastructure for antisense drugs also has the possibility of being less costly, as the same facility may be used to manufacture multiple drugs.

Will antisense oligonucleotides replace small molecule-based therapeutic approaches? Of course not! Compared with small molecule drugs, antisense oligonucleotides have some limitations. Cost is one variable in which small molecule drugs, in general, have an advantage. Although much progress has

been made to reduce the costs of antisense drugs (Deshmukh et al., 2001), they are still more expensive than most small molecule drugs. Second-generation antisense drugs, which are more potent and can be given less frequently, are more attractive as commercial products, as the annual cost of the drug product is beginning to approach that of small molecule drugs. A second limitation for antisense drugs is that current products targeting systemic diseases must be given parenterally, although progress is being made in developing oral formulations of antisense drugs (Agrawal et al., 1995; Geary et al., 2001a). A third limitation, already mentioned, is that the tissue and cellular distribution may not be adequate for treatment of certain diseases, such as neurologic diseases. Continued investments in oligonucleotide chemistries and formulations will broaden the application of the technology and continue to make antisense drugs more attractive as therapeutic agents.

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Specific Inhibition of PTEN Expression Reverses Hyperglycemia in Diabetic Mice

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Signaling through the phosphatidylinositol 3′-kinase (PI3K) pathway is crucial for metabolic responses to insulin, and defects in PI3K signaling have been demonstrated in type 2 diabetes. PTEN (MMAC1) is a lipid/protein phosphatase that can negatively regulate the PI3K pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate, but it is unclear whether PTEN is physiologically relevant to insulin signaling in vivo. We employed an antisense oligonucleotide (ASO) strategy in an effort to specifically inhibit the expression of PTEN. Transfection of cells in culture with ASO targeting PTEN reduced PTEN mRNA and protein levels and increased insulin-stimulated Akt phosphorylation in α-mouse liver-12 (AML12) cells. Systemic administration of PTEN ASO once a week in mice suppressed PTEN mRNA and protein expression in liver and fat by up to 90 and 75%, respectively, and normalized blood glucose concentrations in *db/db* and *ob/ob* mice. Inhibition of PTEN expression also dramatically reduced insulin concentrations in *ob/ob* mice, improved the performance of *db/db* mice during insulin tolerance tests, and increased Akt phosphorylation in liver in response to insulin. These results suggest that PTEN plays a significant role in regulating glucose metabolism in vivo by negatively regulating insulin signaling. *Diabetes* 51:1028–1034, 2002

Phosphatidylinositol 3′-kinase (PI3K) is a crucial signaling enzyme whose activity is regulated by a variety of biological stimuli, including insulin (1). PI3K is composed of two subunits: the p85 regulatory subunit, containing two Src homology-2 domains, and the p110 catalytic subunit (2–4). Binding of insulin to its receptor activates the insulin receptor tyrosine kinase, resulting in autophosphorylation and phosphorylation of several substrates, including insulin receptor substrate (IRS)-1 through -4. IRS then binds to the regulatory subunit of PI3K through its Src homology domains, and this interaction activates the catalytic unit. Activated PI3K phosphorylates the 3′-position of the ring in inositol phospholipids, generating phosphatidylinositol (3,4), diphosphate, and phosphatidylinositol (3,4,5)-triphosphate (PIP3). The lipid products of PI3K initiate phosphorylation and activation of Akt, which is believed to act as a downstream mediator of many of the metabolic effects of insulin (5,6). Thus, the expression of inactive PI3K mutants or chemical agents, such as wortmannin and LY294002 that interfere with PI3K activity, inhibit Akt phosphorylation, glucose uptake, and glycogen and lipid synthesis in vitro (7,8).

PTEN (MMAC1/TEP1) is a dual-specificity protein phosphatase involved in signal transduction and tumor suppression (9,10). PTEN also has phosphoinositide 3′-phosphatase activity and is therefore capable of suppressing PI3K signaling by dephosphorylating PIP3 (11,12). Mutations in PTEN have been associated with several human cancers, and mice heterozygous for the PTEN gene have a high incidence of spontaneous tumors (13,14). Tumor cells and fibroblasts deficient in PTEN have elevated levels of PIP3 and phosphorylated Akt/protein kinase B (PKB) and are resistant to many apoptotic stimuli (15,16).

Because many of the metabolic effects of insulin are mediated through activation of PI3K and the subsequent rise in intracellular PIP3 concentrations, inhibition of a negative regulator of this pathway may enhance insulin signaling. Although the tumor-suppressive functions of PTEN have been elucidated, its physiological role in glucose metabolism in vivo is largely unknown. Inhibition of the *daf-18* gene, a homolog of PTEN in *Caenorhabditis elegans*, can partially bypass the need for DAF-2, an insulin receptor-like molecule (17,18). PTEN overexpression in vitro inhibits glucose uptake and GLUT4 transport in 3T3L1 cells, whereas microinjection of PTEN antibodies increased GLUT4 translocation (19). These results suggest that PTEN may modulate insulin signaling in vivo; however, the lethality of the PTEN null mutation has made this difficult to study. We therefore designed and characterized antisense oligonucleotides (ASOs) targeting PTEN and used them in vitro and in vivo to determine whether the inhibition of PTEN expression affects insulin signaling and glucose metabolism.

RESEARCH DESIGN AND METHODS

Oligonucleotides. A total of 80 oligonucleotides were screened for their ability to inhibit PTEN mRNA expression in T-24 bladder carcinoma cells by quantitative real-time RT-PCR. All oligonucleotides were synthesized as uniform phosphorothioate chimeric oligonucleotides, with 2′-*O*-methoxyethyl groups on bases 1–5 and 16–20. The oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (Perkin Elmer-Applied Biosystems) and purified as described (20). Two active PTEN ASOs complementary to human and mouse PTEN mRNA (Genbank accession nos. AA017584 and AA124728, respectively), a six-base mismatch, and a control

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AS1, antisense oligonucleotide 1; ASO, antisense oligonucleotide; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; IRS, insulin receptor substrate; MIS, mismatch control oligonucleotide; PI3K, phosphatidylinositol 3′-kinase; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKB, protein kinase B; UC, universal control oligonucleotide.

oligonucleotide were used in the experiments described and are designated as follows: antisense oligonucleotide 1 (AS1) (ISIS 116847: 5′-CTGCTAGCCTCT GGATTTGA-3′, beginning at position 2097 in the human RNA); AS2 (ISIS 116845: 5′-CACATAGCGCCTCTGACTGGG-3′, beginning at position 1,539); MIS (ISIS 116848: 5′-CTTCTGGCATCCGGTTTAGA-3′, a six base mismatch to AS1); and UC, a universal control (ISIS 29848: synthesized using a mix of random mixture of A, G, T, and C so that the resulting preparation represents an equimolar mixture of all possible four [19] oligonucleotides).

Cell culture. 3T3L1 murine fibroblasts (American *Type Culture* Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (growth media). Cells were grown to confluence in 12- or 24-well plates before initiating differentiation. Confluent monolayers were differentiated to the adipocyte phenotype by culturing with 500 μmol/l isobutulmethylaxanthine, 250 nmol/l dexamethasone, and 400 nmol/l insulin in growth media for 3 days, followed by growth media alone for 3 days. Following this protocol, >90% of the adipocytes express the fully differentiated phenotype by 6 days after initiation.

Fully differentiated 3T3L1 adipocytes were transfected by the addition of serum-free DMEM and FuGENE6 (Roche) following the manufacturers instructions. The final concentration of 500 nmol/l oligonucleotide and a ratio of 4 μl FuGENE6 per microgram oligonucleotide were empirically determined to maximally suppress target RNA expression. Cell media was typically refreshed 36 h after transfection.

AML12 cells (American *Type Culture* Collection), a nontransformed hepatocyte cell line from transforming growth factor-β transgenic mice, were used to demonstrate antisense-mediated PTEN protein reduction and insulin-stimulated Akt phosphorylation in vitro. The cells were maintained in 90% of 1:1 mixture of DMEM and Ham's F12 medium containing 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% fetal bovine serum. The cells were treated with AS1 or mismatch control oligonucleotide (MIS) for 72 h using Lipofectin (Gibco) as a transfection agent per the manufacturer's instructions. Because AML12 cells require insulin for maintenance, the cells were serum- and insulin-starved for 8 h after transfection, and then 100 nmol/l insulin was added for 30 min before harvesting in lysis buffer for Western blotting.

Northern blots. RNA was prepared from cultured cells using a Qiagen RNA Easy Kit and from animal tissues homogenized in guanidinium isothiocyanate followed by cesium chloride gradients (21). Northern blots were performed as described using full cDNA probes generated by RT-PCR (22). The RNA signal was detected using a PhosphorImager (Molecular Dynamics) and normalized against the signal for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) using ImagePro software.

Western blots. Cells or tissues were harvested in lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris, pH 7.5, 1% Triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l ortho-vanadate, 1 mmol/l NaF, and 1:200 dilution of protease cocktail III; (Calbiochem), and the proteins were separated by SDS-PAGE. After transfer of the proteins onto polyvinylidine fluoride membranes, the blots were reacted with antibodies to phospho-Akt (New England Biolabs), Akt (New England Biolabs), or PTEN and developed using enhanced chemiluminescence (Amersham Pharmacia).

Mice and metabolic measurements. All animal experiments were performed under the institutional American Association for the Accreditation of Laboratory Animal Care (AALAC) guidelines. Male *db/db* mice (C57BLKS/J-*m* +/+*Lepr^{db}*) and age-matched lean littermates (C57BLKS/J-*m*+/+ *Lepr^{db}*) at 10 weeks of age or male *ob/ob* (C57BL/6J-*Lep^{ob}*) at 8 weeks of age (The Jackson Laboratory) were used for all experiments. Mice were maintained on a 12-h light/dark cycle and fed ad libitum unless otherwise noted. Whole blood was obtained from the retro-orbital sinus of fed mice, and glucose was measured using a Metabolics glucose oxidase-based analyzer. Mice were weighed once a week, and food intake was monitored over a 24-h period. An insulin tolerance test was performed after a 4-h fast by intraperitoneal injection of 1 unit/kg human insulin (Lilly). Blood was drawn from the tail before insulin injection (time 0) and then 30, 60, and 90 min afterward and measured as described above. For in vivo phospho-Akt measurements, mice were fasted for 12 h and then injected with 2 units/kg insulin.

Serum glucose, triglycerides, and cholesterol concentrations were analyzed on a Johnson and Johnson Vitros 950 automated clinical chemistry analyzer, and serum insulin concentrations were quantitated using an enzyme-linked immunosorbent assay for rat insulin (Alpco).

RESULTS

Characterization of PTEN ASO in vitro. ASOs designed to be complementary to human and mouse PTEN genomic sequences were screened for suppression of PTEN mRNA expression in cells in vitro, as previously described (23). The most potent oligonucleotide from the screen, AS1 (see RESEARCH DESIGN AND METHODS), reduced PTEN mRNA levels in a concentration-dependent manner in 3T3-L1 adipocytes. Similar results were obtained with a second PTEN antisense (AS2) that hybridizes to a different region of the PTEN mRNA. A control antisense with six mismatched nucleotides (MIS) to AS1 was inactive in reducing PTEN mRNA expression. Maximal inhibition (90%) of PTEN protein expression was achieved after 72 h of oligonucleotide treatment, which is presumably indicative of the intrinsic half-life of the PTEN protein (data not shown).

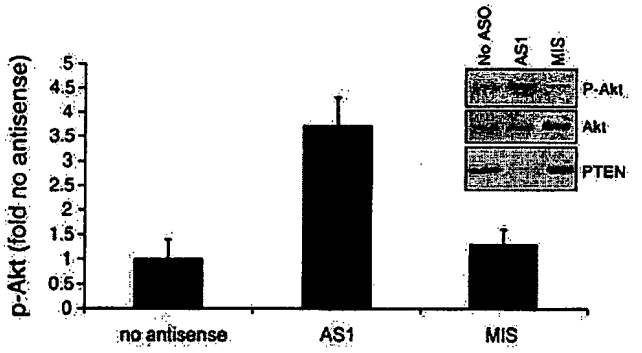

FIG. 1. Reduction of PTEN protein levels and stimulation of Akt phosphorylation by PTEN antisense treatment in vitro. AML12 cells were treated with AS1 or MIS for 72 h using Lipofectin as a transfection agent. Immunoblots of cell proteins were sequentially reacted with antibodies to phospho-Akt, -Akt, and -PTEN. Data are expressed as the fold increase in phospho-Akt intensity compared with Lipofectin-only treated cells. Graphs represent the mean of three replicates ±SD.

As described above, PIP3 is believed to initiate phosphorylation and activation of Akt, an important downstream mediator of the metabolic effects of insulin (24). We reasoned that if PTEN is indeed involved in modulating insulin signaling, inhibition of PTEN expression might increase the level of Akt phosphorylation in response to insulin. To test this, AML12 cells were treated with PTEN AS1, and the effects on PTEN protein levels and insulin-stimulated Akt phosphorylation were examined. Cells treated with PTEN AS1 resulted in a >90% reduction in PTEN protein levels (Fig. 1). Furthermore, PTEN AS1 treatment resulted in an increase in insulin-stimulated phosphorylation of Akt by ~3.5-fold, relative to untreated and MIS-treated cells (Fig. 1), whereas the Akt protein levels remained the same.

Antisense-mediated inhibition of PTEN expression in vivo. Based on the in vitro results obtained with AS1, we reasoned that inhibition of PTEN expression might improve insulin sensitivity in the *db/db* mouse, a rodent model of type 2 diabetes. First, we investigated the ability of systemically administered AS1 to reduce PTEN mRNA and protein levels in insulin-sensitive tissues. The half-life of 2′-*O*-methoxyethyl chimeric phosphorothioate oligonucleotides is ~7 to 19 days in the liver, depending on the dose (25). Therefore, *db/db* mice were treated by intraperitoneal injection once a week for 4 weeks with 10, 25, or 50 mg/kg of AS1, and PTEN mRNA levels in liver, fat, and muscle tissues were measured by Northern blotting. PTEN mRNA levels were reduced in a dose-dependent manner in

liver extracts from treated mice relative to saline controls, with maximal inhibition occurring (88%) at the 50-mg/kg dose (Fig. 2*A*). In lean littermates dosed with 100 mg/kg of AS1, PTEN mRNA levels were also reduced by >90%, relative to saline-treated controls. There was no apparent difference in the relative levels of PTEN mRNA in untreated lean versus *db/db* mice. Moreover, neither the MIS nor another control oligonucleotide, universal control oligonucleotide (UC), affected PTEN mRNA levels significantly. Also, the mRNA levels of PTP1B and SHIP2, two other phosphatases that have the potential to inhibit insulin signaling (26,27), were not affected by PTEN AS1 treatment (Fig. 2*B*). These results demonstrate that the effect of AS1 was both PTEN target-specific and antisense sequence-specific and indicate that the metabolic effects of the PTEN antisense (described below) were primarily caused by a specific reduction in PTEN expression.

PTEN protein levels in liver samples from saline-, AS1-, and UC-treated *db/db* mice were analyzed by Western blotting. After 4 weeks of AS1 treatment, a dose-dependent decrease in PTEN protein levels in livers from *db/db* mice was observed (Fig. 2*C*). Reduction of PTEN protein levels was also observed in livers from lean littermates treated with AS1. As with the mRNA results, no difference in the relative levels of PTEN protein in control lean versus *db/db* mouse livers was apparent, nor were any effects observed by a control oligonucleotide (UC) on PTEN protein levels.

Northern analysis of other insulin-sensitive tissues demonstrated that PTEN mRNA levels were also reduced in a dose-dependent manner, relative to saline controls, in fat tissue from AS1-treated *db/db* mice, with maximal inhibition of 80% at the 50 mg/kg dose (Fig. 2*D*). A similar reduction in PTEN protein levels in fat from AS1-treated mice was also observed (data not shown). PTEN message levels appeared to be less abundant in muscle relative to liver and fat, and no consistent reduction in PTEN mRNA expression was observed in the skeletal muscle of animals treated with PTEN AS1. This result is in agreement with pharmacokinetic studies showing that accumulation of oligonucleotides in muscle after parenteral injection is relatively low (28). Interestingly, no PTEN protein was detectable on immunoblots of muscle lysates (data not shown).

Effect of inhibiting PTEN expression on glucose, insulin, and lipid concentrations in diabetic and lean mice. Having characterized the effect of AS1 on PTEN mRNA and protein expression in vivo, we next investigated the effect of inhibiting PTEN expression on hyperglycemia in *db/db* and *ob/ob* mice. Blood glucose concentrations in *db/db* mice were reduced in a dose-dependent manner over the course of a 4-week treatment with AS1 (Fig. 3*A*), becoming normalized (138 ± 5 mg/dl) at the highest dose tested (50 mg/kg). The second PTEN antisense, AS2, produced a similar reduction in serum glucose levels at the end of 4 weeks of treatment (Fig. 3*B*). In related studies, treatment of *db/db* mice with PTEN AS2 resulted in a reduction of PTEN mRNA and protein levels in liver that was comparable with that produced in animals treated with PTEN AS2 (data not shown). In contrast, neither the MIS nor UC controls affected glucose levels. Furthermore, PTEN antisense treatment had no effect on glucose concentrations in lean littermates, despite the fact

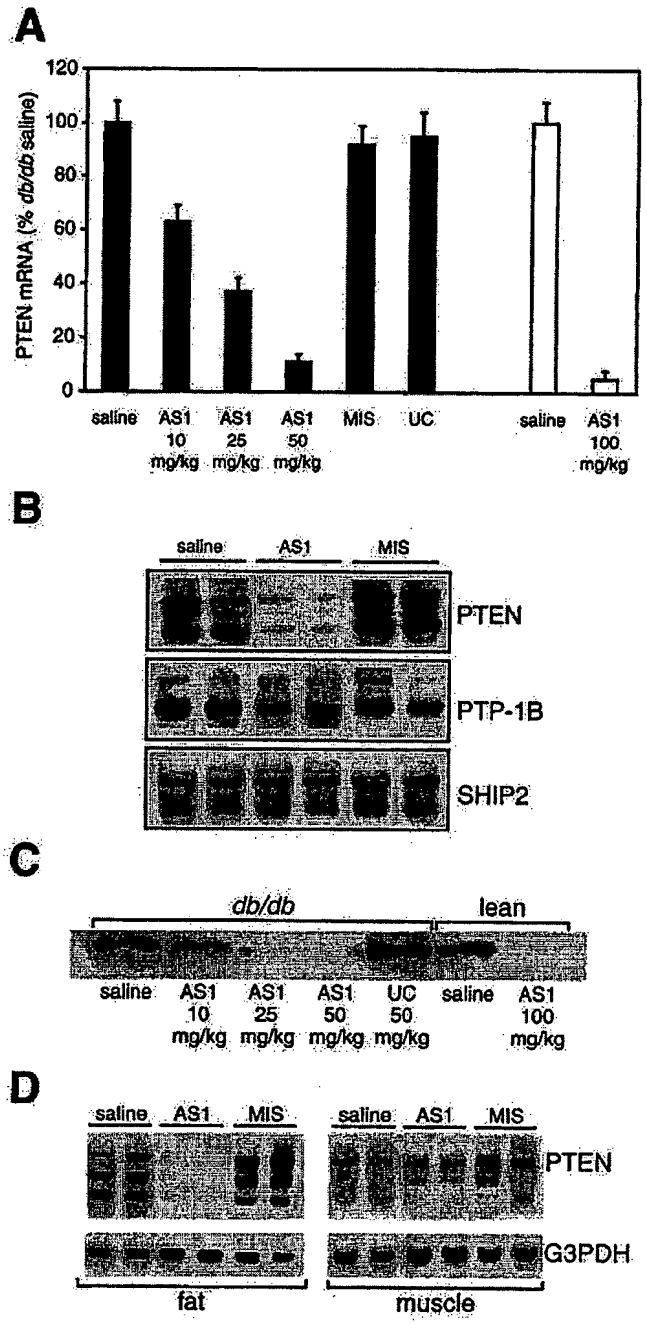

FIG. 2. PTEN antisense specifically reduces PTEN mRNA and protein levels in livers and fat from *db/db* mice. *A*: Dose-dependent reduction of PTEN mRNA levels in liver. *db/db* (black bars) and lean (white bars) mice received indicated doses of ASOs intraperitoneally once a week for 4 weeks. Total mRNA was prepared from liver and was analyzed by Northern blotting (n = 3 per group). The PTEN signal was normalized against the signal for G3PDH. Data are expressed as the mean percentage of mRNA levels in saline-treated *db/db* mice ±SD. *B*: Specificity of PTEN antisense. Representative Northern blots of PTEN mRNA (*A*), PTP1B mRNA (*B*), and SHIP2 mRNA (*C*) in livers from *db/db* mice treated once a week for 4 weeks with saline, 50 mg/kg AS1, or 50 mg/kg MIS. Each lane contained 25 μg of RNA from an individual animal. *C*: Reduction of PTEN protein expression in liver. PTEN immunoblots of proteins in liver lysates from mice treated for 4 weeks with indicated doses of AS1 or UC. Each lane contained 50 μg of protein. *D*: Reduction of PTEN mRNA in fat but not muscle. Representative Northern blots of PTEN and G3PDH mRNA in fat and muscle from *db/db* mice treated with saline, 50 mg/kg AS1, or 50 mg/kg MIS.

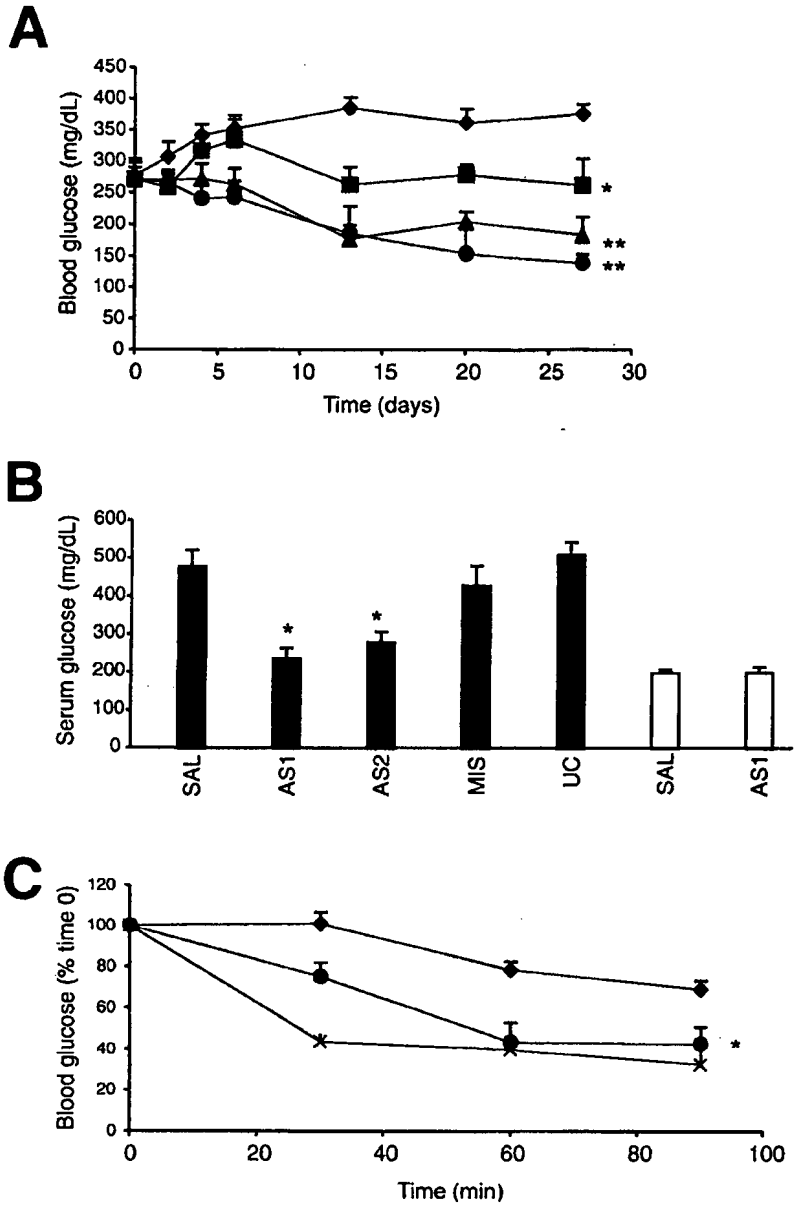

FIG. 3. Inhibition of PTEN expression lowers glucose and increases insulin sensitivity in *db/db* mice. *A*: Time- and dose-dependence of glucose-lowering effects of PTEN AS1. *db/db* mice were injected intraperitoneally once a week with saline (♦) or 10 mg/kg (■), 25 mg/kg (▲), or 50 mg/kg(●) of AS1 in saline. Mice were bled every 2 days for the first week and then once a week thereafter, 6 days after the previous dose. Values are expressed as means ± SE (n = 6–8). Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, **P < 0.0001, *P < 0.01. *B*: Specificity of the antidiabetic effect of PTEN ASOs on serum glucose concentrations. *db/db* mice (■, n = 5–6) were injected intraperitoneally with 50 mg/kg of indicated oligonucleotides for 4 weeks. Lean littermates (□, n = 5) were dosed with saline or 100 mg/kg of AS1. Statistics were performed using ANOVA, followed by Bonferroni-Dunn. Compared with saline-treated, *P < 0.005. *C*: Insulin tolerance test in PTEN antisense–treated mice. Mice were treated once a week for 3 weeks with saline (♦) or 50 mg/kg AS1 (●) (n = 5). Lean controls (x) were untreated. Results are expressed as the mean ±SE percentage of the glucose concentration at time 0. Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, *P < 0.05.

that PTEN mRNA and protein levels were reduced to the same extent as that of *db/db* mice.

To determine whether inhibition of PTEN expression had an effect on insulin sensitivity, an insulin tolerance test was performed in *db/db* mice treated once a week for 3 weeks with saline or AS1 (Fig. 3*C*). PTEN AS1 significantly increased sensitivity to insulin; the relative blood glucose concentrations in the AS1-treated mice were significantly lower at all time points after insulin injection compared with those in saline-treated animals. Also, inhibition of PTEN expression did not appear to cause hypoglycemia, in that glucose levels in mice remained normoglycemic in PTEN AS1–treated *db/db* and lean mice fasted for 16 h (data not shown).

Inhibition of PTEN expression also lowered serum triglyceride and cholesterol concentrations in *db/db* mice in a dose-dependent manner (Table1). Lipid concentrations were unaffected relative to lean littermates at the 50-mg/kg dose of AS1, and the control oligonucleotide had no effect. Treated *db/db* mice gained significantly more weight than saline- and control antisense–treated *db/db* mice, despite the fact that food intake was similar in all groups. However, *ob/ob* mice did not gain weight relative to saline-treated animals during their 4-week antisense treatment (see below). No significant changes in body composition (i.e., lean versus fat body mass) were observed in any mice treated with PTEN ASOs (data not shown).

The effect of inhibiting PTEN expression on hyperinsulinemia was investigated in *ob/ob* mice, which have higher circulating levels of insulin and are less hyperglycemic than *db/db* mice. Male *ob/ob* mice were injected with 50 or 20 mg/kg of AS1 on day 0 and then with either 20 or 10 mg/kg a week thereafter for 3 weeks, a dosing schedule designed to attain more moderate steady-state levels of oligonucleotide in liver. At the end of 4 weeks, PTEN

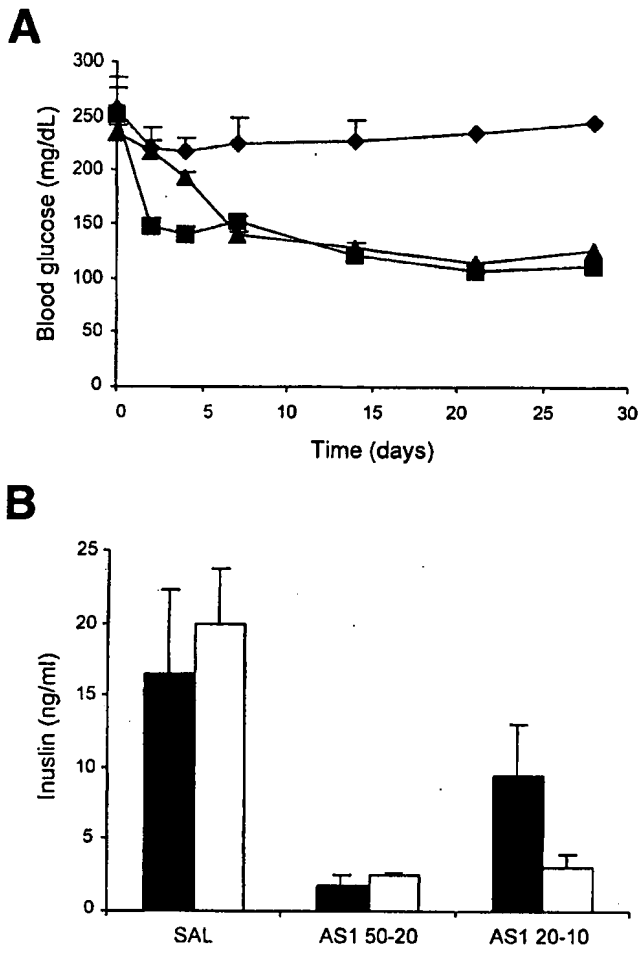

FIG. 4. Reduction of blood glucose (*A*) and serum insulin (*B*) in *ob/ob* mice treated with PTEN antisense. *A*: *Ob/ob* mice were injected intraperitoneally once a week with saline (♦) or 50 mg/kg AS1 on day 0, followed by 20 mg/kg once a week thereafter (■), or 20 mg/kg AS1 on day 0, followed by 10 mg/kg once a week thereafter (▲). Values are expressed as means ± SE (n = 8). *B*: *ob/ob* mice treated as described above were fasted for 4 h before measuring serum insulin after 2 weeks (■) and 4 weeks (□) of dosing. Values are expressed as the means ± SE (n = 6).

mRNA levels were reduced by 71% in the higher-dose group and by 49% in the lower-dose group (data not shown) relative to saline-treated animals, and blood glucose concentrations were normalized by 2 weeks in both dose groups (Fig. 4*A*). Serum insulin levels were reduced by 90% at 2 weeks in the higher-dose group and by 84% at 4 weeks in the lower-dose group (Fig. 4*B*).

Effect of inhibiting PTEN expression on Akt phosphorylation in diabetic and lean mice. If the effects of PTEN inhibition on glucose and insulin levels in diabetic mice are caused by an activation of PI3K signaling, evidence of a biochemical improvement in insulin signaling downstream of PI3K should be detectable. Because Akt activation is dependent on the products of PI3K, we reasoned that decreasing PTEN expression would result in increased levels of Akt phosphorylation in response to insulin in diabetic animals. To test this, *ob/ob* mice and their lean littermates were treated with either saline, AS1, or MIS at 50 mg/kg once a week for 2 weeks, and PTEN and phospho-Akt protein levels were determined in liver.

As can be seen in Fig. 5*A*, PTEN protein expression was reduced by ~90% in livers from both lean and *ob/ob* mice treated with AS1, relative to saline-treated mice. In lean mice, neither the basal levels of Akt phosphorylation nor the sixfold increase in Akt phosphorylation in response to a bolus insulin injection were affected by PTEN antisense treatment (Fig. 5*B*). As has been previously reported in diabetic rats (29), no increase in Akt phosphorylation in response to insulin was observed in control-treated *ob/ob* mice. However, PTEN AS1 treatment appeared to restore Akt phosphorylation in response to insulin in *ob/ob* mice (Fig. 5*C*). However, PTEN AS1 treatment did not appear to affect basal levels (non–insulin-stimulated) of phosphorylated Akt in *ob/ob* mice.

DISCUSSION

The molecular defects that cause insulin resistance and hyperglycemia in type 2 diabetes have not been well defined. Impaired insulin receptor function leading to reduced activation of PI3K could be a cause of insulin resistance, or the primary defect may lie further downstream in the PI3K pathway. The results presented here indicate that PTEN, a tumor suppressor with phosphoinositide 3′-phosphatase activity, may play a role in glucose metabolism in vivo by negatively regulating insulin signaling.

Several lines of evidence indicate that PI3K activation and the subsequent rise in PIP3 concentrations are necessary for many of the metabolic responses to insulin, including Akt activation, glucose transport, and glycogen and lipid synthesis. PTEN is capable of dephosphorylating PIP3 (12), and cells in which PTEN activity has been inhibited have elevated PIP3 concentrations and higher levels of Akt phosphorylation (16). Thus, it seems logical that PTEN can regulate insulin signaling through the PI3K pathway. Indeed, Nakashima et al. (19) recently demonstrated that overexpression of PTEN in 3T3-L1 cells inhibits glucose uptake and GLUT4 translocation in vitro, whereas microinjection of a PTEN antibody increased basal and insulin-stimulated GLUT4 translocation also in vitro. Our results demonstrating that antisense-mediated reduction of PTEN expression increased insulin-dependent Akt phosphorylation in vitro also supports the conclusion that PTEN may negatively regulate insulin signaling in cultured cells.

Moreover, using our antisense approach, we were able to determine the effect of inhibiting PTEN expression in animals, and our results suggest that PTEN plays an important role in glucose homeostasis in vivo as well. We have shown that systemic administration of PTEN antisense reduced PTEN mRNA and protein expression in a dose-dependent manner in mouse liver but had no effect on the levels of the phosphatases PTP1B and SHIP2. Similar results have recently been obtained using a Fas ASO, which reduced Fas mRNA and protein expression in hepatocytes by up to 90% after systemic injection in mice (25). We also found that systemically administered PTEN oligonucleotides are capable of reducing target expression in fat, but not muscle, and that PTEN mRNA and protein levels are much less abundant in muscle than in fat and liver. Taken together, these results indicate that the effects of the antisense were indeed specific for PTEN and suggest that the reduction of PTEN expression primarily in

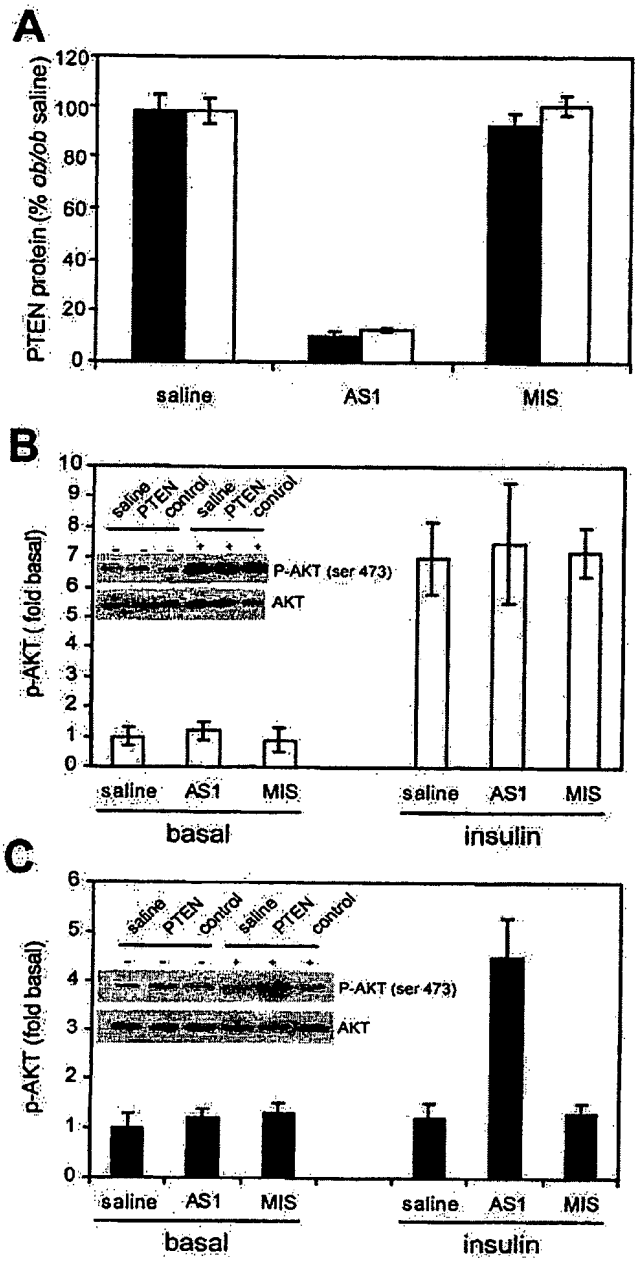

FIG. 5. Reduction of PTEN expression in *ob/ob* and lean mice and increased Akt phosphorylation in *ob/ob* mice treated with PTEN antisense. *A*: Reduction of PTEN protein levels in both *ob/ob* (■) and lean (□) mouse livers after PTEN antisense treatment. Mice (n = 4 per group) were injected for 2 weeks with saline or 50 mg/kg ASI or MIS oligonucleotides, and liver lysates were immunoblotted with antibodies to PTEN and G3DPH. Data are expressed as the mean percentage of normalized PTEN protein levels in saline-treated *ob/ob* mice ±SD. *B* and *C*: Phospho-Akt levels in livers of lean and *ob/ob* mice without (basal) or 5 min after intraperitoneal injection of 2 units/kg of insulin. Immunoblots of proteins were sequentially reacted with antibodies to phospho-Akt and Akt. Data are expressed as the fold increase in phospho-Akt intensity compared with basal levels in saline-treated mice relative to Akt protein levels. Graphs represent the means ± SD. Representative gels of pooled samples (n = 3) from each group are also shown for lean and *ob/ob* mice.

liver, with some possible contribution in fat, was sufficient to produce the observed metabolic changes in diabetic mice. Improved insulin sensitivity in liver of diabetic mice would be expected to reduce hepatic glucose production and thereby reverse hyperglycemia.

Antisense-mediated inhibition of PTEN expression normalized glucose concentration in both *db/db* and *ob/ob* mice, improved insulin sensitivity in *db/db* mice, and lowered insulin concentrations dramatically in *ob/ob* mice. The fact that inhibition of PTEN expression reversed hyperglycemia and reduced insulin resistance in diabetic mice, without affecting glucose levels in lean mice, suggests that the reduction in PTEN expression compensated for a defect in the PI3K pathway in diabetic mice. Alternatively, it is possible that inhibition of PTEN in diabetic mice may somehow compensate for defects in other pathways that are unrelated to PI3K but may contribute to insulin resistance in these animals. No detectable difference in PTEN mRNA or protein levels in lean versus *db/db* mice was observed, so it does not appear that an increase in PTEN expression levels is the primary defect in these mice. It has previously been demonstrated that IRS-associated PI3K activity is decreased significantly in *ob/ob* mouse liver (30) and that Akt/PKB activity is reduced in liver and muscle from diabetic rats and humans (29). We have demonstrated that inhibition of PTEN expression in vivo restores insulin-stimulated Akt phosphorylation in *ob/ob* diabetic mice to a level comparable with that in lean mice. We have also observed significant increases in liver Akt phosphorylation in *db/db* mice treated with PTEN antisense (data not shown). Thus, it seems logical that a reduction in PTEN expression after antisense treatment resulted in increased PI3K activity by increasing the half-life and/or effective concentration of PIP3 produced during insulin activation. This logic is consistent with the putative role of PTEN in PI3K signaling, as well as with the results of other investigators who have employed different approaches for suppressing PTEN activity in vitro (11–14,17–19). Nevertheless, we cannot rule out the possibility that the effects of PTEN inhibition on Akt phosphorylation and insulin sensitivity that we have observed in vitro and in vivo may not be directly related to increased PI3K activity and increased PIP3 levels because neither of these end points were measured directly. Although PTEN antisense treatment had no effect on insulin-dependent Akt phosphorylation or on circulating glucose levels in lean mice, serum insulin levels were decreased by 50%, suggesting that PTEN inhibition may increase insulin sensitivity in lean mice as well. Interestingly, a recent report about a Cowden's disease patient with a heterozygous PTEN mutation indicated improved insulin sensitivity, as measured by glucose clearance and hyperinsulemic-euglycemic clamp (31). However, although several groups have demonstrated an increased incidence of tumors in PTEN heterozygous mice, no changes in blood glucose concentrations have been reported. This finding may not be surprising in view of the fact that in our studies, reducing PTEN expression by 90% had no significant effect on glucose levels in lean mice.

In conclusion, our results demonstrate that suppression of PTEN expression produces a marked improvement in blood glucose concentrations and insulin sensitivity in diabetic mice and suggest that pharmacological inhibition of negative regulators of the PI3K pathway may represent

a therapeutic approach for the treatment of type 2 diabetes.

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5

Methods of Selecting Sites in RNA for Antisense Targeting

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I. INTRODUCTION

The mechanism of action for antisense oligonucleotides requires that the oligonucleotide hybridize to its mRNA target. Therefore, in principle, design of an antisense oligonucleotide requires simply that the oligonucleotide be complementary to the mRNA. In practice, however, when several oligonucleotides complementary to an mRNA are screened, certain antisense oligonucleotides are more active and more potent than others in suppressing specific gene expression (1–12). In addition, some complementary oligonucleotides can show nonantisense effects (13–15). The most commonly used and most effective approach to discovery of antisense oligonucleotides involves synthesis of numerous oligonucleotides (up to several dozen) designed to hybridize to different regions of the targeted mRNA, followed by activity screening in cells (16).

Several attempts have been made to identify features of oligonucleotides that are associated with antisense activity. Development of successful methods for selection of active oligonucleotides prior to oligonucleotide synthesis and cell-based screening would have two benefits. First, the cost of antisense discovery would be reduced. If a computer algorithm could pick the most active compound for an antisense target, then synthesis and screening of multiple compounds could be eliminated. Second, identification of the features associated with specific and nonspecific effects of oligonucleotides would likely lead to a better understanding of the detailed mechanism of antisense activity and, potentially, to identification of compounds with even greater potency. This review will discuss methods that have been used to select antisense oligonucleotides, the

effectiveness of these methods, and the prospect for improved methods in the future.

II. RNA STRUCTURE CALCULATIONS

It has long been assumed that activity of an antisense oligonucleotide is directly related to the hybridization affinity of the oligonucleotide for its mRNA target. Support for this assumption comes from the observation that, at a given target site, longer oligonucleotides are more active than shorter ones (17). In addition, at a given site, oligonucleotide modifications that increase the melting temperature (T_m) of the oligonucleotide-RNA duplex, often increase antisense activity and/or potency (18–21). Mismatched oligonucleotides reduce the T_m and decrease the potency (22,23).

However, when comparing oligonucleotides targeted to different sites, T_m alone is not sufficient to ensure activity (3). It has long been believed that secondary structure in the mRNA target affects hybridization affinity differently at different sites and thus affects antisense efficacy (24–28). Therefore, methods for calculating RNA structure and calculating hybridization of the antisense oligonucleotide to the structured mRNA are useful for prediction of antisense activity.

Early attempts by Stull et al. (29) found moderate correlation (R = 0.66–0.99) between a predicted duplex score and antisense activity. Inclusion of an mRNA target secondary-structure score in the calculation actually worsened correlation between calculated hybridization affinity and antisense activity. Since Stull's publication, improvements have been made to the rules and parameters for prediction of RNA secondary structure. (30). Effective parameters for prediction of DNA:RNA duplex stability are available (31) and improved parameters for prediction of secondary structure in DNA oligonucleotides are also available (32–37). Mathews et al. (38) used these most up-to-date parameters to calculate equilibrium affinity of complementary DNA or RNA oligonucleotides to an RNA target taking into account the predicted stability of the oligonucleotide-target helix and the competition with predicted secondary structure of both the target and the oligonucleotide. When their predicted affinities were compared to antisense activity in one experiment (39), good correlation (R = 0.91) was found between duplex free energy and antisense activity. When oligonucleotide self-structure and/or target RNA structure were included in the calculation, antisense efficacy did not correlate with $\Delta G°_{overall}$.

The reported correlations between predicted duplex stability and antisense activity do not extend broadly to additional targets. When a data set of 349 antisense oligonucleotides targeting 12 genes (Giddings and Matveeva, *http://antisense.genetics.utah.edu*) was evaluated for correlation between duplex stability and antisense activity, the linear correlation coefficient was 0.22, suggesting that the strong correlations reported in earlier work do not extend to larger data sets.

There are several possible explanations for the lack of a strong correlation between calculated hybridization of an oligonucleotide to its mRNA target and observed antisense activity. One possibility is that the calculated binding energies do not represent true equilibrium affinities. Although current algorithms are good enough to correctly predict 73% of base pairs in structures determined from comparative sequence analysis (30), this level of accuracy may not be enough to allow prediction of good antisense-binding sites. In addition, current algorithms (38) use thermodynamic parameters for unmodified DNA or RNA when calculating free energies of antisense: RNA duplex formation or antisense oligonucleotide self-structure. Parameters determined from experiments using modified oligonucleotides could improve the predictions (40). Furthermore, parameters for predictions were measured in 1 M Na^+, 0.1 mM EDTA, and may not represent conditions of antisense binding. The large numbers of proteins involved in RNA synthesis, processing, transport, translation, and degradation almost certainly affect binding of the antisense oligonucleotide to its target.

A second possibility is that the antisense target is pre-mRNA and secondary structures predicted for mRNAs are not representative of structures in pre-mRNAs. It is known that pre-mRNA is the molecular target for many antisense oligonucleotides (41,42). The secondary structure of a pre-mRNA undergoing synthesis, processing, and transport is likely not fully predictable from simple thermodynamic consideration.

The third, and most likely, possibility is that equilibrium affinity is not the sole factor impacting antisense activity (43). Oligonucleotide sequence and structure may affect properties of the antisense compound such as its affinity for proteins, ability to support RNAse H cleavage of the target, delivery to the cellular site of activity, and metabolic stability. These factors will, in turn, affect antisense activity. On the other hand, equilibrium affinity is not unimportant. When oligonucleotide sequence is kept constant, mRNA secondary structure affects antisense activity in a predictable way; activity is lower in structured targets than in unstructured ones (44).

Although factors other than target structure clearly play a role in antisense activity, predictions of local secondary structure have proven effective in identifying oligonucleotides with greater activity than those found by simple oligonucleotide "walks." The strategy employed by Sczakiel and colleagues (45,46) searches for favorable local target elements, loops or bulges of ~10 nt, joints and terminal sequences. Although successful application of this strategy to other targets has not yet been reported, it is tempting to speculate that the success is due to the fact these favorable local target elements represent kinetically preferred sites. "Kissing" hairpins are known to be important for initiation of hybridization

of long antisense RNAs (47,48); these "favorable structures" may play a similar role for oligonucleotide hybridization.

III. OLIGONUCLEOTIDE MOTIF PREFERENCES

It has been suggested that active oligonucleotides contain certain sequence motifs. Tu et al. (49) report that TCCC is associated with antisense activity but no mechanism for this phenomenon was proposed. Smetsers et al. (50) previously reported that CCC is overrepresented in the antisense oligonucleotides in their data set but that TCC is underrepresented. They suggest that overrepresented motifs may be associated with protein-binding and nonantisense effects. Lesnik and Freier (51) offered a plausible explanation for the predominance of pyrimidines and especially C's in active oligonucleotides. They suggests that antisense activity is associated with high stability of the oligo: target hybrid *relative to the alternative RNA:RNA duplex*. Thus antisense oligodeoxyribonucleotides with high (70–80%) pyrimidine content and moderate (40–50%) (A + T) content are more likely to be active than oligonucleotides with different composition.

IV. CELL-FREE SCREENING AND COMBINATORIAL APPROACHES

Several groups have described combinatorial approaches for identification of optimal antisense sites in target mRNA using a cell-free assay. Typically, a library of randomized oligonucleotides is incubated with the target mRNA and RNase H. Mapping of the most favored RNase H cleavage sites results in identification of the most favored binding sites. This approach has been used to find sites for both antisense oligonucleotides (39,52–54) and ribozymes (55). It can, however, be complicated by interactions of library oligonucleotides with each other and by binding of multiple oligonucleotides to the mRNA target (56). Concerns over library complexity have limited oligonucleotide lengths in these studies to 10 nt. Optimal binding sites for short oligonucleotides may not predict those for longer, antisense oligonucleotides.

Matveeva et al. (57) were able to use longer oligonucleotides and reduce library complexity by restricting the oligonucleotide pool to oligonucleotides complementary to the mRNA target sequence. A similar, but less thorough screen was performed by Jarvis et al. (58), who used a cell-free RNase H assay with individual oligonucleotides to identify optimal sites for synthetic ribozymes.

Optimal binding sites have also been identified without using RNase H cleavage assays. Ecker et al. (13) screened randomized combinatorial libraries of 2′-*O*-methyl- and phosphorothioate-modified compounds and identified compounds that bind to H-*ras* mRNA. Using oligonucleotide arrays on glass slides, Southern and colleagues (59,60) were able to identify compounds that bound tightly to c-raf mRNA and were able to select the site for ISIS 5132, the most potent c-raf antisense compound reported at that time. Their synthetic approach uses an elegant strategy that results in synthesis of only oligonucleotides complementary to the mRNA of interest.

The effectiveness of these cell-free approaches requires that the most favored site(s) for oligonucleotide binding to the mRNA in the cell-free system will be the target site for the most active antisense oligonucleotide. To test whether this was the case, Matveeva et al. (61) evaluated the correlation between activity in an RNase H mapping assay or a gel shift binding assay with antisense activity in cells. Moderate correlation with cellular activity ($R = 0.6$) was found for both cell-free assays. Similar correlation analysis of the randomized library data of Ho (39,52) and the array data of Mir (62) gave coefficients of correlation between activity in the cell-free assay and antisense activity ranging from 0.2 to 0.7 (O. Matveeva, J. Wyatt, and S. Freier, unpublished). Thus the correlation between activity in the cell-free assay and antisense activity is relatively weak.

Despite the relatively weak correlation observed between oligonucleotide binding in the cell-free assay and antisense activity, ribozymes (55) or antisense oligonucleotides (39,52,54,57) designed to sites identified by combinatorial selection were more likely to be active than those selected without initial cell-free screening. Thus these methods can improve the "hit rate" for antisense discovery. However, these methods are cumbersome and, at best, result in several leads that still need to be screened in a cell-based assay. Therefore, the benefit of improved hit rate may not make up for the substantial cost disadvantage associated with these cell-free combinatorial assays.

V. SPECIFICITY CONSIDERATIONS AND NONANTISENSE EFFECTS

A. Target Specificity

One attraction of antisense technology is that high specificity can be achieved. For example, inhibition of one isoform of a protein can be obtained without affecting another (11,63,64). Such specificity is hard to achieve with small-molecule drugs. To obtain such specificity, one must be careful to design antisense oligonucleotides that will not hybridize to related mRNA sequences (65). Since oligonucleotides with as few as three mismatches are reported to be inactive (23), three mismatches to related targets should be sufficient but more would be desirable.

Unfortunately, the most commonly used tool for identification of sequence

homology, BLAST (66), is ineffective at finding mismatched sites for oligonucleotides. This is because the default window size is 11, meaning that there must be 11 matches in a row for BLAST to find the homology. The window size can be set as small as 7, but even then 20-mers with two mismatches (for example, in positions 7 and 14) are not found. A more effective technique for finding mismatched sites is to use BLAST to identify other mRNA sequences with homology to the target of interest and then use a substring search to find mismatched sites in these mRNAs. Sites with zero or a few mismatches should be avoided.

B. Motifs that Support Nonantisense Effects

Nonantisense effects of G-rich phosphorothioate oligonucleotides are well known (13,14) and have been attributed to the tendency of these oligonucleotides to form G-quartet structures that then interfere with biological processes (67). The simplest way to avoid these effects is to avoid G-rich oligonucleotides. Restricting oligonucleotides to less than 50% G with no G_4 strings and at most one G_3 string usually does not detrimentally limit the number of oligonucleotides that can be selected from a target message.

Homopolymers of other sequences also form unusual structures (68). Although nonantisense effects of these structures are not well characterized, this should be considered when designing oligonucleotides rich in any single nucleotide or containing strings of any single nucleotide.

Other motifs are also reported to produce nonantisense effects. Krieg et al. (15) reported that oligonucleotides containing CG, especially those with RRCGYY, can stimulate murine B cells in vitro and in vivo. The active motif in human cells is GTCGTT (69). To avoid designing any oligonucleotides containing the dinucleotide CG is, however, an overly stringent requirement. It eliminates nearly half the possible oligonucleotides that hybridize to a typical message from consideration, many of which show no immune stimulation at all. Therefore, it may be more prudent to avoid oligomers with the consensus hexamer motifs or to restrict the number of CGs in the sequence to less than two. In addition, the immunostimutatory effects of CG motifs are easily eliminated by chemical modification (e.g., 5-methyl C) (70).

VI. OTHER CONSIDERATIONS

A. Cross-Species and Cross-Isoform Oligonucleotides

One feature of antisense inhibitors is that usually an active inhibitor of the human target is not an inhibitor of the same gene in mouse or another species. This is because mRNA sequences differ between species. It is sometimes possible, however, to select sites with high identity between two species and design oligonucleotides to those sites. If a sufficient number of such sites are tested, it may be possible to identify an antisense oligonucleotide with activity in both species. Similarly, if sufficient sequence identity exists between two isoforms, it may be possible to identify an antisense oligonucleotide with activity against both targets. Using this strategy an oligonucleotide with good activity against both JNK-1 and JNK-2 was identified (71).

B. Target Site Function

The preceding discussion has considered RNA secondary structure at the target site and oligonucleotide sequence but has not seriously addressed position of the target site on the mRNA relative to functional sites such as the coding region. This is because antisense oligonucleotides that operate by an RNAse H mechanism seem to be affected little by target site function. Potent oligonucleotides have been reported for the coding regions, untranslated regions, and even introns. On the other hand, antisense oligonucleotides that use a non-RNase H mechanism are typically restricted to specific functional sites. Morpholino oligonucleotides, for example, inhibit via translation arrest and are often located near or upstream of the AUG initiation codon (72). They can also inhibit splicing if placed at splice junctions (21). Thus target site function becomes more important if a "steric blocking" mechanism of action is employed.

VII. SUMMARY

Design of antisense oligonucleotides is, in principle, very simple. In practice, on the other hand, only a fraction of antisense oligonucleotides complementary to an RNA target are active. Computational predictions of hybridization affinity that take into account RNA target structure, oligonucleotide self-structure, and oligonucleotide-RNA hybridization have had limited success at identifying potent antisense sites. Cell-based screening of a number of compounds is still required. Combinatorial approaches offer the potential of finding the best antisense oligonucleotide for any target. These approaches have not, in general, identified compounds with substantially greater activity than those designed by more conventional methods. In addition, significant effort is required for the cell-free screen and several compounds must still be screened in cell-based assays. Although no single approach has yet provided a method for identifying the single best target site for an antisense oligonucleotide, several guidelines are listed here that may improve "hit rates" and avoid screening of compounds likely to have nonantisense activities.

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6

Pharmacokinetic Properties in Animals

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I. INTRODUCTION

Antisense technology is a fundamentally different approach to disease treatment both because it provides a simple and rational approach to drug discovery and because it targets a new biological template, mRNA. The mRNA target represents an earlier and, by definition of the antisense method, a more selective target for interrupting the translation of proteins that are involved in the cause or maintenance of disease.

The unique pharmacology of antisense oligonucleotides requires intense study of their in vivo pharmacokinetics. In addition, to aid in the characterization of the safety of antisense oligonucleotides, their exposure as a function of dose and ultimately the rates and mechanisms of clearance from the body must be defined. The development of these compounds as therapeutics has received increasing attention in recent years. As is often the case with new therapeutics and chemistries, the lack of sensitive and selective bioanalytical methods precludes the use of unlabeled materials. Therefore, many of the studies characterizing animal and human pharmacokinetics have relied on radiolabel tracer experiments. Over the past few years, however, methods that provide selective, sensitive, and reliable quantitation of oligonucleotides in biological fluids (1–6) and tissues (7) have allowed characterization of unlabeled material and provided information on metabolism of these compounds. These methods have greatly facilitated the advance of our understanding of antisense phosphorothioate oligonucleotide pharmacokinetics. The objectives of this review are to provide a summary of more recent progress in animal pharmacokinetics and to provide an integrated understanding of the absorption, distribution, metabolism, and excretion of phosphoro-

PTP1B antisense oligonucleotide lowers PTP1B protein, normalizes blood glucose, and improves insulin sensitivity in diabetic mice

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The role of protein-tyrosine phosphatase 1B (PTP1B) in diabetes was investigated using an antisense oligonucleotide in *ob/ob* and *db/db* mice. PTP1B antisense oligonucleotide treatment normalized plasma glucose levels, postprandial glucose excursion, and HbA_{1C}. Hyperinsulinemia was also reduced with improved insulin sensitivity. PTP1B protein and mRNA were reduced in liver and fat with no effect in skeletal muscle. Insulin signaling proteins, insulin receptor substrate 2 and phosphatidylinositol 3 (PI3)-kinase regulatory subunit p50α, were increased and PI3-kinase p85α expression was decreased in liver and fat. These changes in protein expression correlated with increased insulin-stimulated protein kinase B phosphorylation. The expression of liver gluconeogenic enzymes, phosphoenolpyruvate carboxykinase, and fructose-1,6-bisphosphatase was also down-regulated. These findings suggest that PTP1B modulates insulin signaling in liver and fat, and that therapeutic modalities targeting PTP1B inhibition may have clinical benefit in type 2 diabetes.

Insulin resistance in peripheral tissues including liver, fat, and skeletal muscle characterizes type 2 diabetes. Insulin resistance is associated with other pathological conditions including obesity, hypertriglyceridemia, and hypertension (1). The molecular mechanism of insulin resistance is not well understood but does not appear to involve structural defects in the insulin receptor (IR) (2). The molecular basis for insulin resistance appears to involve an early process in IR signal transduction (3, 4).

Insulin signaling is initiated when insulin binds to the extracellular α-subunits of the $\alpha_2\beta_2$ heterotetrameric insulin receptor. Insulin binding to the IR induces activation of the kinase domain and autophosphorylation of at least six tyrosine residues within three distinct domains of the β-subunit (5). Autophosphorylation of tyrosine residues 1146, 1150, and 1151 in the kinase domain of the β-subunit activates the insulin receptor kinase and causes the phosphorylation of other protein substrates, including IR substrates (IRS-1 to -4) and other adapter proteins (Gab1 and Shc) that mediate the biological effects of insulin (6–9).

Protein-tyrosine phosphatase 1B (PTP1B) negatively regulates IR and IRS-1 phosphorylation (10–12). Mice that have the PTP1B gene ablated have increased insulin sensitivity with resistance to weight gain on a high-fat diet (13, 14). However, the role of PTP1B in the diabetic state has not been elucidated. To determine the effect of reducing PTP1B protein level in the diabetic state and subsequent effect on insulin signaling, an antisense oligonucleotide (ASO) was optimized to selectively bind to PTP1B mRNA and reduce PTP1B protein expression (15, 16). PTP1B ASO was demonstrated to improve glycemic control in obese and insulin resistant diabetic *ob/ob* and *db/db* mice. In *ob/ob* mice, PTP1B ASO was also demonstrated to affect the expression of insulin signaling proteins in liver and fat and to improve insulin sensitivity in liver. These data suggest that PTP1B modulates insulin signaling in liver and fat and that therapeutic modalities targeting PTP1B inhibition may have clinical benefit in type 2 diabetes.

Experimental Procedures

Selection of PTP1B ASO. Rapid throughput screens for identifying ASO inhibitors selective against PTP1B were performed with 20-base chimeric ASOs where the first five bases and last five bases have a 2′-*O*-(2-methoxy)-ethyl (2′-MOE) modification. The 2′-MOE modification increases binding affinity to complementary RNA sequences and increases resistance to nucleases (17). The ASO oligonucleotides have a phosphorothioate backbone and use an RNase H-dependent mechanism for activity. Initial screens were conducted against rat PTP1B and ten ASOs were identified as hits, all of which targeted the same binding site within the coding region of the PTP1B mRNA. Subsequently, a series of *in vitro* characterization experiments were performed in primary rat and mouse hepatocytes, in which ISIS-113715 was consistently identified to be the most potent and specific oligonucleotide in reducing PTP1B mRNA levels. ISIS-113715 hybridizes to PTP1B mRNA at nucleotides 861–880 in the coding sequence. A universal control (UC) oligonucleotide pool (ISIS-29848) was synthesized as a mixture of A (adenine), G (guanosine), T (thymine), and C (cytosine) bases so that the resulting preparation contains an equimolar mixture of all 4^{19} possible oligonucleotides. The oligonucleotide chemistry of ISIS-29848 was identical to that of ISIS-113715.

Animal Care and Treatments. *ob/ob* and *db/db* mice and their lean littermates of 6–7 weeks of age (The Jackson Laboratory) were acclimated to the animal research facilities for 5 days. The following investigations were conducted in accordance with each institutions IACUC guidelines. Animals were housed five per cage *ob/ob* (C57BL/6J- Lep^{ob}/Lep^{ob}), two per cage lean *ob/+* littermates (C57BL/6J-Lep^{ob}/+), four per cage *db/db* (C57BLKS/J-*m*$Lepr^{db}/Lepr^{db}$), and two per cage lean *db/+* littermates (C57BLKS/J-*m*$Lepr^{db}$/+) and maintained on mouse

Abbreviations: ASO, antisense oligonucleotide; PTP1B, protein-tyrosine phosphatase 1B; PI3, phosphatidylinositol 3; IR, insulin receptor; IRS, IR substrate; PKB, protein kinase B; PEPCK, phosphoenolpyruvate carboxykinase; ITT, insulin tolerance test; GTT, glucose tolerance test; UC, universal control.

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chow (*ob/ob* Labdiets #5015, St. Louis; *db/db* Harlan-Teklad rodent diet #8604 Madison, WI; 26% fat calories) *ad libitum*.

After acclimation the *ob/ob* and lean mice were weighed and tail snip glucose levels were determined by the glucose oxidase method (Precision G glucose meter, Abbott Laboratories, North Chicago). The animals were randomized to the various treatment groups based on plasma glucose levels and body weight. Baseline plasma insulin samples were taken from a subset of the animals representing each treatment group once randomized (n = 10 *ob/ob* and n = 10 lean *ob/+* littermates; ELISA, ALPCO Diagnostics, Windham, NH). Three separate experiments were performed with PTP1B ASOs. Treatment groups were as follows. Experiment 1: *ob/ob* and *ob/+* mice treated with PTP1B ASO at 50 mg/kg (n = 9) for 3 weeks; experiment 2: *ob/ob* and *ob/+* mice treated with PTP1B ASO at 25, 2.5, or 0.25 mg/kg or saline (n = 10 per treatment) for 6 weeks; experiment 3: *db/db* and *db/+* mice treated with PTP1B ASO at 50, 25, or 10 mg/kg or saline or universal control oligonucleotide (UC) at 50 mg/kg for 4 weeks (n = 8 per treatment glucose and n = 3 per treatment for all other parameters). Mice were dosed i.p. either twice per week (*ob/ob* and *ob/+*) or once per week (*db/db* and *db/+*). The ASO were weighed and resuspended in saline at a concentration of 25 mg/ml. The suspension was vortexed and allowed to sit at room temperature for 15 min and was then filtered through a syringe filter (0.2 μm; Gelman Acrodisc). The filtrate (2 μl) was diluted in 1 ml of H_2O and OD read at 260 nM. The formula used to calculate the concentration was as follows: (OD · dilution factor · molecular weight)/(extinction coefficient · 1,000) = concentration in mg/ml. The stock was diluted to the desired concentration for injection in sterile saline and frozen at 20°C. For subsequent use, the stock was thawed, heated to 37°C, and vortexed before using. At the end of each week tail bleed glucose and insulin (*ob/ob* and *ob/+* only) levels, as well as body weight, were determined under nonfasting conditions by 10:00 a.m. (as described above). A gross estimation of food consumption was determined in *ob/ob* mice each week as follows. Food was measured at 10:00 a.m. at the start and end of a 24-h period (same 24-h period each week), and divided by the number of mice per cage for an index of estimated 24 h food consumption per mouse. At the end of the studies, liver, epididymal fat pads, and skeletal muscle were harvested and frozen immediately in liquid nitrogen for further analysis.

Glucose and Insulin Tolerance Tests (GTT and ITT). An i.p. GTT was performed at 0.5 g/kg (50% solution of D-50 Dextrose, Abbott Laboratories, North Chicago). After a 3-h fast beginning at 6:30 a.m., a baseline 0-min sample was taken followed by an i.p. injection of glucose. Tail bleed samples were taken at 15, 30, 60, and 120 min following glucose injection. *ob/ob* and *ob/+* mice were studied after 3 or 6 weeks of treatment. The 6-week 25 mg/kg and saline control treatments underwent an i.p. ITT (2 units insulin per kg in 0.1% BSA; R-Insulin, Lilly Research Laboratories, Indianapolis). After a 5-h fast and a baseline 0-min tail bleed for glucose determination, an i.p. injection of insulin was given and additional glucose samples were taken at 15, 30, 60, and 120 min.

Insulin Challenge. In a parallel group of identically treated *ob/ob* mice, a saline or insulin challenge was administered at 0 min after an overnight fast. Insulin (2 units/kg in 0.1% BSA) or saline control was given i.p. Tissue samples from liver (0, 1, and 5 min) were taken under both saline and insulin stimulated conditions (n = 4 per treatment per time point). Within each challenge (saline and insulin) were subgroups of saline- or antisense-treated (25 mg/kg) mice.

Tissue Extract Preparation, Immunoprecipitation, and Immunoblotting Techniques. Tissues were sonicated (using a Branson 450 Sonifier) in lysis buffer containing 20 mM Tris·HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 10 μg/ml leupeptin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 1 mM microcystin and rocked for 40 min at 4°C. Detergent-insoluble material was sedimented by centrifugation at 12,000 × g for 10 min at 4°C. Cell lysate proteins (50 μg of protein) were separated by SDS/PAGE on 10% and 7.5% gels or 100 μg of protein was immunoprecipitated for 2 h with 4G10 antiphosphotyrosine antibodies (4 μg/ml; Upstate Biotechnology, Lake Placid, NY). Immune complexes were collected with protein A-Sepharose, washed, solubilized in Laemmli sample buffer, and separated by SDS/PAGE on 7.5% gels. Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% milk. The blots were probed with various primary antibodies—antiphosphotyrosine, anti-PTP1B, anti-IRS-1 (PH domain), anti-IRS-2, anti-p85 antibodies (Upstate Biotechnology, Lake Placid, NY), anti-IRβ antibody (Transduction Laboratories, San Diego), and phospho-protein kinase B (PKB) antibody (New England Biolabs)—according to the recommendations of the manufacturers. The proteins were detected by enhanced chemiluminescence with horseradish peroxidase-labeled secondary antibodies (Amersham Pharmacia). The intensity of the bands was quantitated with a laser densitometer (Molecular Dynamics).

RNA Preparation. RNA preparation was performed by grinding approximately 100 mg of liver tissue in 1 ml of TRIzol reagent and analysis was done according to the Affymetrix protocol. Briefly, the RNA from four mice in PTP1B ASO-treated or control groups was pooled using equal amounts to make a total of 20 μg of RNA. cRNA was prepared using the Superscript Choice system from GIBCO/BRL Life Technologies (no.18090-019). The protocol was followed with the exception that the primer used for the reverse transcription reaction was a modified T7 primer with 24 thymidines at the 3' end. The sequence was 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGG-AGGCGG-$(T)_{24}$-3'. Following this, labeled cRNA was synthesized according to the manufacturer's instructions from the cDNA, using the Enzo RNA Transcript Labeling Kit (no. 900182). Approximately 20 μg of cRNA was then fragmented in a solution of 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc at 94°C for 35 min. Labeled cRNA was hybridized to the Affymetrix GENECHIP Test2 Array to verify the quality of labeled cRNA. Following this, cRNA was hybridized to the Affymetrix MU11K A and B chip. The cRNA was hybridized overnight at 45°C. The data were analyzed using GENECHIP version 3.2 and SPOTFIRE.NET Version 5.0 (Spotfire, Somerville, MA). The microarray experiment was repeated for the 50 mg/kg treatment group by using RNA isolated a second time from the same mouse livers, and the results are an average of the two experiments.

Statistical Analysis. Statistical evaluation was performed via 1-way ANOVA and t tests where appropriate, using INSTAT (GraphPad, San Diego). The level of significance was $P < 0.05$ (two-sided test).

Results and Discussion

PTP1B ASO was administered to obese, insulin-resistant *ob/ob* and *db/db* mice. Diabetic *ob/ob* mice were treated i.p. twice per week for 6 weeks in a dose-ranging study. PTP1B protein levels were reduced in liver and fat without a reduction in skeletal muscle (Fig. 1 *A–C*). In the high-dose group, hepatic PTP1B mRNA levels were also reduced (44 ± 2%, $P < 0.05$). This is

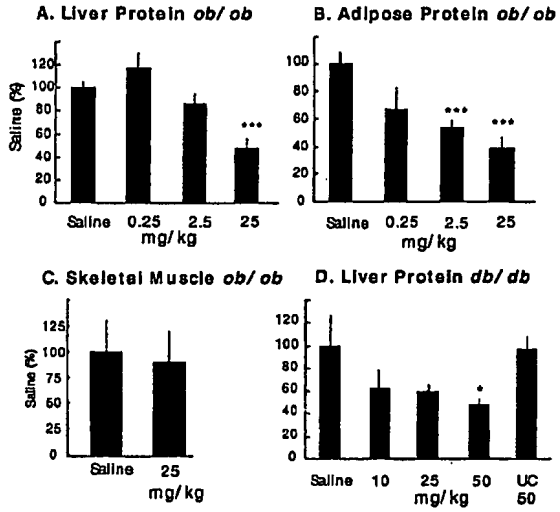

Fig. 1. PTP1B protein levels in liver (*A*), fat (*B*), and skeletal muscle (*C*) from *ob/ob* mice treated i.p. for 6 weeks twice per week with PTP1B ASO at the indicated dose. (*D*) Liver PTP1B protein levels in *db/db* mice treated i.p. once per week for 4 weeks at the indicated dose. In the *db/db* study, a UC oligonucleotide dosed at 50 mg/kg was included to control for nonspecific effects of the PTP1B ASO. Data are mean ± SE and statistics were determined as a two-tailed *t* test. *, $P < 0.05$; ***, $P < 0.001$ (vs. saline control).

consistent with the known accumulation of ASOs into liver and fat with little penetration into muscle (18). The same PTP1B ASO lowered PTP1B protein (Fig. 1*D*) and mRNA levels (55 ± 8%, $P < 0.05$) in liver of diabetic *db/db* mice treated i.p. weekly for 4 weeks. A UC combinatorial mixture of oligonucleotides (see *Experimental Procedures*) was without effect (Fig. 1*D*) on PTP1B levels in liver.

After 2 weeks of treatment, plasma glucose levels in *ob/ob* mice were corrected to lean (*ob/+*) levels in the 25 mg/kg dose group and by week 3 were improved in the 2.5 mg/kg dose group (Fig. 2*A*). After 6 weeks of treatment, an overnight fast reduced glucose levels (30%) in the high-dose group (139 ± 14 vs. 97 ± 3 mg/dl, $P < 0.05$) with no hypoglycemia. HbA_{1C}, a measure of long-term glucose homeostasis, was reduced from 6.2 ± 0.3% in saline-treated *ob/ob* mice to 4.7 ± 0.1% ($P < 0.01$) in the 25 mg/kg dose group, a level equivalent to lean *ob/+* littermates (4.8 ± 0.1%). Plasma insulin levels were decreased 77% (Fig. 2*B*) at 6 weeks in the 25 mg/kg-dose group versus the saline-control group. As demonstrated in an i.p. GTT, glucose excursion was corrected to that of lean animals (*ob/+*) in the 25 mg/kg treatment group and improved in the other two dose groups (Fig. 3*A*). Although *ob/+* mice are lean and not diabetic, they are insulin-resistant and have an impaired glucose tolerance compared with wild-type C57BL/6J mice. The PTP1B ASO-treated lean *ob/+* group also had a statistically significant improvement in glucose excursion, with no observed hypoglycemia (Fig. 3*A*). In *ob/ob* mice, an enhanced reduction in glucose level (3.4-fold; Fig. 3*B*) occurred during an ITT with PTP1B ASO treatment. The GTT and ITT results suggest enhanced insulin sensitivity in adipose and liver because PTP1B ASO treatment reduced PTP1B protein levels in liver and adipose tissue with no effect in skeletal muscle. In *db/db* mice, glucose levels were improved in a dose-dependent manner, reaching lean (*db/+*) littermate levels at 50 mg/kg ASO (Fig. 2*C*). No change in plasma glucose in *db/db* mice was observed with 50 mg/kg UC treatment and no effect on glucose level was observed with either PTP1B ASO or UC treatment in *db/+* mice (data not shown). PTP1B ASO treatment was well tolerated in all animals.

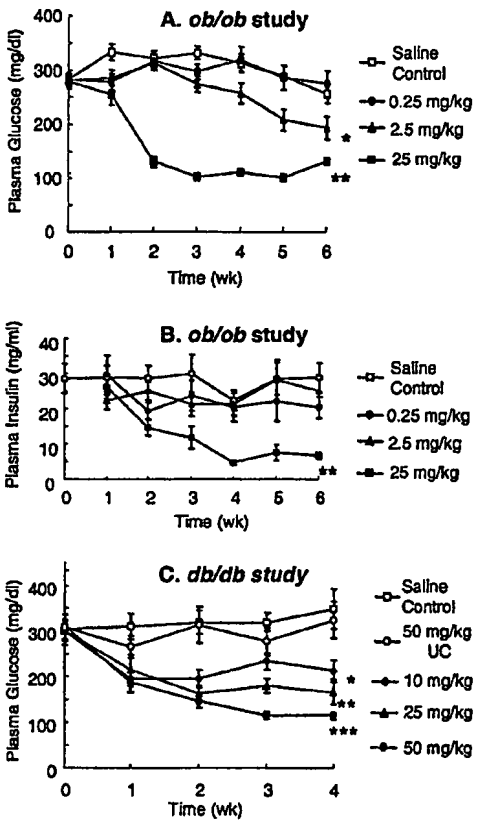

Fig. 2. Non-fasting plasma glucose (*A*) and insulin (*B*) levels versus time in PTP1B ASO-treated *ob/ob* mice (25, 2.5, and 0.25 mg/kg). Nonfasting plasma glucose (*C*) levels versus time in PTP1B ASO-treated *db/db* mice (50, 25, 10, and 50 mg/kg UC). Data are mean ± SE; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. saline control). In *A*, the significance of week 3–6 values dosed at 2.5 mg/kg is $P < 0.05$, and for week 2–6 values dosed at 25 mg/kg is $P < 0.01$. In *B*, the significance of week 3–6 values dosed at 25 mg/kg is $P < 0.01$. In *C*, the significance of week 2–4 values dosed at 10 mg/kg is $P < 0.05$, at 25 mg/kg is $P < 0.01$, and at 50 mg/kg PTP1B ASO is $P < 0.001$.

Molecular toxicology, blood chemistry, and histological examination indicated that PTP1B ASO treatment at these doses did not adversely affect liver function or the general health of the *ob/ob* mice in these studies.

Non-fasted body weights were not different between saline-control and ASO treatment at 0.25, 2.5, and 25 mg/kg over the duration of the 6-week study (Table 1). Weekly weight gain was not different between treatments (data not shown). Epididymal fat weight was reduced 42% with high-dose treatment ($P < 0.05$, normalized to brain weight) and unchanged with lower dose treatments versus saline control (Table 1). Liver weight was increased 36% with 25 mg/kg PTP1B ASO treatment only ($P < 0.05$, normalized to brain weight; Table 1). It is possible that metabolism of lipids was altered such that flux through utilization pathways (and/or diminished storage) was enhanced resulting in reduced fat weight. Future studies are needed to determine the meaning and mechanism of this observation in this model. Estimated food consumption was decreased in the 25 mg/kg PTP1B ASO-treated *ob/ob* mice compared with the saline controls during week 6 (4.9 ± 0.3 vs. 5.9 ± 0.3 g/day; $P < 0.05$; 25 mg/kg PTP1B ASO versus saline). There were no differences in food intake during weeks 1, 2, and 5 ($P > 0.05$). Consequently, reduction in weight gain and food intake is

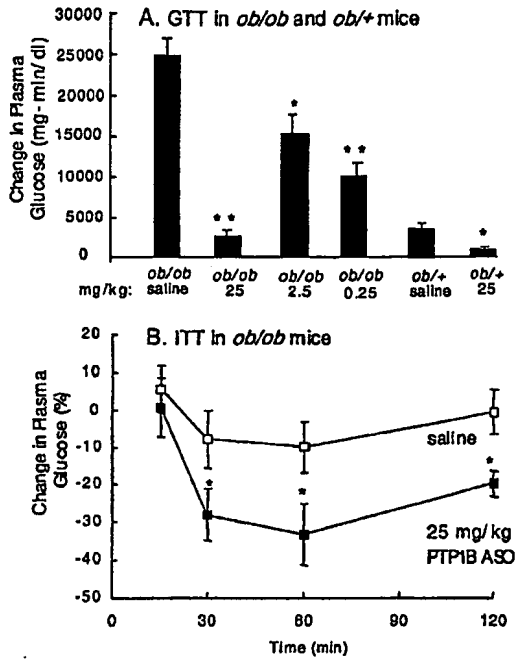

Fig. 3. PTP1B ASO treatment increases insulin sensitivity in *ob/ob* mice. (*A*) Change in AUC (area under the curve) for plasma glucose after an i.p. GTT. (*B*) Change in plasma glucose level after an ITT in *ob/ob* mice. Results are expressed as change from baseline $AUC_{Glucose}$ for GTT (25, 2.5, and 0.25 mg/kg PTP1B ASO treatment in *ob/ob* and 25 mg/kg PTP1B ASO treatment in lean littermates, *ob/+*). Results are expressed as percentage change from baseline for ITT. GTT and ITT were performed in week 6 of treatment. Data are mean ± SE; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. saline control).

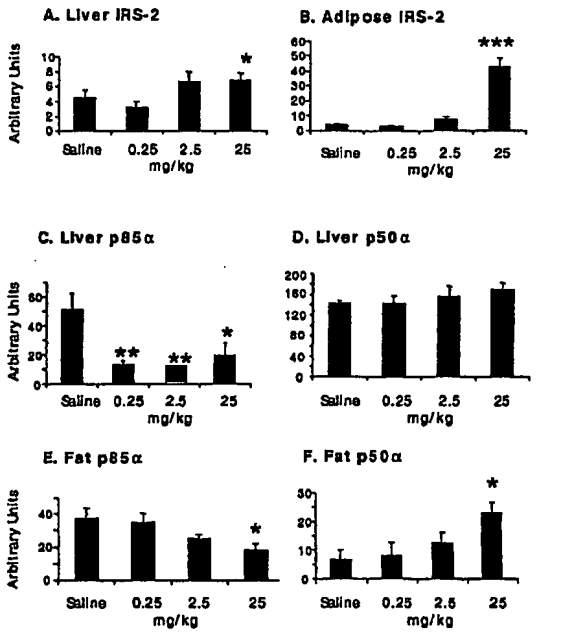

Fig. 4. PTP1B ASO treatment affects the level of IRS-2 and PI3-kinase regulatory subunit (p85α and p50α) expression in *ob/ob* mouse liver and fat. Representative immunoblots using anti-IRS-2 antibodies (*A* and *B*) or anti-p85α whole antiserum that recognized all p85 isoforms (*C–F*) were quantified. The results are the average of four mice within each group. The data are represented as arbitrary units and are the mean ± SEM. Statistics were determined as a two-tailed *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. saline control).

unlikely to be responsible for the normalization of glucose and the decrease in insulin levels. Examination of the *ob/ob* mice in these studies for changes in histopathology, blood chemistry, and molecular toxicology did not reveal any adverse reaction to treatment with the PTP1B ASO.

Stimulation of IR kinase activity (19, 20) induces the tyrosine phosphorylation of endogenous substrates including IRS-1 to -4 (9). IRS proteins interact with and recruit SH2 domain-containing proteins, including phosphatidylinositol 3 (PI3-kinase; refs. 21 and 22). In *ob/ob* diabetic mice, the expression levels of IR, IRS-1, and IRS-2 are reduced relative to lean littermates, and may contribute to the insulin-resistant phenotype (23). Although in gene deletion experiments PTP1B has been shown to be an important regulator of IR and IRS-1 in nondiabetic mice (13, 14), it is not clear that a decrease in PTP1B expression will regulate proteins involved in the insulin-signaling pathway in the diabetic state. We therefore measured the protein expression of IR, IRS-1, IRS-2, and PI3-kinase isoforms in liver and fat from *ob/ob* and *ob/+* mice treated with PTP1B ASO by immunoblotting. No effect on IR or IRS-1 expression was detected in liver or fat (data not shown) from PTP1B-ASO treated mice, although reduced expression of IR, IRS-1, and IRS-2 was confirmed in *ob/ob* relative to *ob/+* mice, as reported (23). Reduced levels of IRS-2, considered important in hepatic insulin signal transduction (24), could contribute to hepatic insulin resistance and increased IRS-2 expression could improve hepatic insulin sensitivity. Although a direct causality cannot be established, IRS-2 protein levels were increased in a dose-dependent manner in liver and fat in *ob/ob* mice treated for 6 weeks with PTP1B ASO (Fig. 4 *A* and *B*). PTP1B ASO treatment had no effect on IRS-2 levels in the same tissues from lean *ob/+* mice (data not shown).

IRS-1 and IRS-2 link IR to its final actions through a series of intermediate effectors. One of the key enzymes downstream of metabolic IR signaling is PI3-kinase (21, 22, 25). PI3-kinase is a heterodimeric enzyme composed of a regulatory subunit (p85α) and a catalytic subunit (p110). Additional isoforms of p85α (e.g., p55α and p50α) have also been described (25). Mice with targeted disruption of the gene encoding the p85α subunit of PI3-kinase have increased levels of p55α and p50α, and exhibit increased insulin sensitivity and hypoglycemia (26). The change in isoform expression is associated with increased insulin-induced phosphatidylinositol 3,4,5-triphosphate formation, increased GLUT4 translocation, and increased glucose transport into fat and muscle. To determine whether insulin sensitization was associated with a change in PI3-kinase expression, homog-

Table 1. Body, epididymal fat, and liver weights (g) after 6 weeks of saline or PTP1B antisense treatment (2qw, i.p.) in nonfasted *ob/ob* mice

	Body weight	Epididymal fat	Liver
Saline	58.1 ± 1.3	4.9 ± 0.1	4.0 ± 0.1
0.25 mg/kg	59.1 ± 0.8	5.0 ± 0.3	3.6 ± 0.2
2.5 mg/kg	59.3 ± 1.4	4.2 ± 0.3	4.5 ± 0.2
25 mg/kg	56.4 ± 1.1	3.0 ± 0.2***	5.6 ± 0.3**

Data are mean ± SE. Epididymal fat and liver weights were normalized to brain weight for each animal for statistical purposes. There were no differences between treatments in brain weight ($P > 0.05$). **, $P < 0.01$; ***, $P < 0.001$ vs. saline control. At randomization on day 1, body weights did not differ between groups (37.1 ± 0.6, 37.6 ± 0.8, 37.5 ± 0.8, and 37.2 ± 0.7 g; saline control, 0.25, 2.5, and 25 mg/kg PTP1B ASO treatment, respectively).

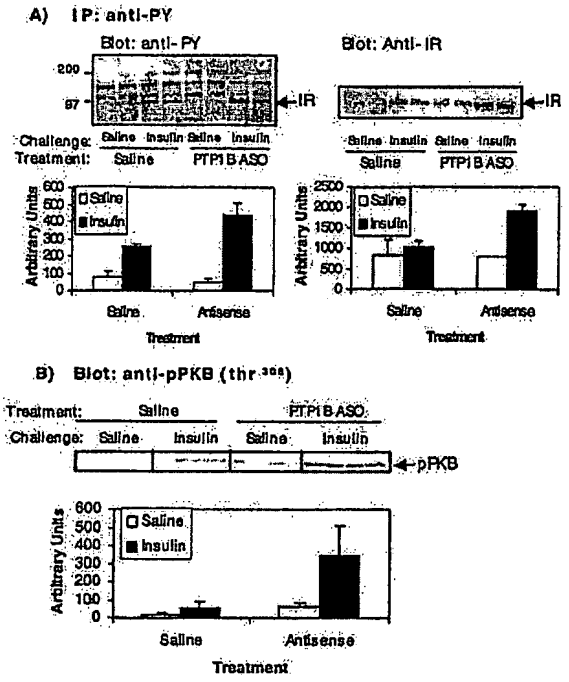

Fig. 5. PTP1B ASO causes enhanced insulin signaling in liver. PTP1B ASO (25 mg/kg, i.p. dosed twice per week for 6 weeks) or saline-treated *ob/ob* mice were fasted for 5 h and then challenged with an i.p. bolus of saline or insulin (2 units/kg in 0.1% BSA). Liver tissue was taken at 1 min following saline or insulin challenge. (*A*) Cell lysates were immunoprecipitated using anti-phosphotyrosine (PY) antibodies and immunoblotted with anti-PY and anti-IR antibodies as indicated. (*B*) Cell lysates were loaded by equal amount of proteins, separated by SDS/PAGE (7.5% gels), and immunoblotted with anti-phosphothreonine-308-specific PKB antibodies.

enates of liver and fat from PTP1B ASO-treated lean and obese animals were immunoblotted with p85α antiserum. A dose-dependent reduction of p85α isoform expression in both liver and fat was observed along with increased expression of the p50α isoform in fat (Fig. 4). In a 3-week study in *ob/ob* mice dosed at 50 mg/kg i.p., twice per week with PTP1B ASO, a similar phenotype was observed with decreased expression of p85α in liver (40%, $P < 0.04$) and fat (30%, $P < 0.01$). In the same study, increased expression of both p50α, 2-fold in liver ($P < 0.01$) and 20-fold in fat ($P < 0.01$), and p55α, 6-fold in liver ($P < 0.01$), was observed (data not shown). No changes in PI3-kinase isoform expression were observed in skeletal muscle, supporting the notion that the changes were not secondary to a generalized improvement in glucose tolerance and insulin sensitivity (data not shown). Differential expression of PI3-kinase regulatory subunits observed in the PTP1B ASO-treated *ob/ob* mice would be predicted to increase insulin sensitivity in liver and fat and is consistent with the improved glucose tolerance observed in these diabetic mice.

To determine whether PTP1B ASO enhanced insulin signaling we investigated the effect of the PTP1B ASO treatment on insulin-dependent IR tyrosine phosphorylation (Fig. 5*A*). An i.p. insulin challenge (2 units/kg) was performed in *ob/ob* mice previously treated for 6 weeks with saline or 25 mg/kg PTP1B ASO. Livers were extracted at 1 min post insulin or saline challenge. An approximate 2-fold increase in the insulin-dependent tyrosine phosphorylation of the insulin receptor was observed in the PTP1B ASO-treatment animals compared with saline-treated animals. PKB is thought to regulate some IR metabolic responses downstream of IRS and PI3-kinase (27, 28). Treatment with PTP1B ASO slightly increased basal PKB phosphothreonine-308 levels. However, ASO treatment caused a much greater enhancement (≈4-fold) in insulin-stimulated PKB Thr-308 phosphorylation (Fig. 5*B*). PKB protein levels were unchanged by PTP1B ASO treatment (data not shown). The increased phosphorylation of PKB is further evidence of increased insulin sensitivity in the liver as a result of PTP1B ASO treatment, and correlates with changes in the expression of insulin signaling proteins IRS-2 and PI3-kinase isoforms.

Hepatic glucose production has a prominent role in systemic glucose homeostasis. Insulin decreases hepatic glucose output by activating glycogen synthesis and glycolysis and by inhibiting gluconeogenesis. Several reports have suggested that PKB is a regulator of gluconeogenesis and glycogen synthesis in liver (29–32). Insulin regulates glycogen synthesis and glycolysis presumably through a PI3-kinase-dependent pathway (21). Insulin also inhibits glucagon and glucocorticoid-induced phosphoenolpyruvate carboxykinase (PEPCK) expression acting through PI3-kinase (33, 34). PEPCK is a rate-limiting step in hepatic gluconeogenesis that is regulated at the transcription level and fructose-1,6-bisphosphatase (F-1,6-BP) is also a key enzyme in this pathway. To investigate the effect of reduced PTP1B protein levels on these downstream events in the hepatic insulin signaling pathway, we measured mRNA levels by microarray analysis (Affymetrix murine 11K chip) comparing PTP1B ASO-treated to saline-treated diabetic *ob/ob* mice. PTP1B ASO significantly reduced PEPCK mRNA levels (38%) and F-1,6-BP (17%) in *ob/ob* mice treated for 6 weeks at 25 mg/kg. A similar reduction was observed for both PEPCK (55%) and F-1,6-BP (52%) mRNA in an independent *ob/ob* mouse study dosing i.p. twice per week for 3 weeks with 50 mg/kg PTP1B ASO.

Reduction of PTP1B protein levels in the diabetic state by using the PTP1B ASO positively modulates insulin-signaling proteins in liver and fat. Increased insulin sensitivity and PKB activation in liver could cause the observed decrease in PEPCK mRNA levels and associated decrease in hepatic glucose output. Decreased hepatic glucose output, as well as a putative increase in insulin sensitivity in fat, could account for the observed improvement in glucose control in PTP1B ASO-treated *ob/ob* mice. These results suggest that a relative over-activity of PTP1B is important to the maintenance of the diabetic state and that inhibition of PTP1B activity, as with a PTP1B ASO-induced decrease in PTP1B protein expression, may provide therapeutic benefit for the treatment of type 2 diabetes.

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